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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The E3 ubiquitin ligase, hRPF1/Nedd4 has been previously been described as a potentiator of progesterone receptor (PR)- and p53-dependent transcriptional activity. Given the observation that hRPF1/Nedd4 shares amino acid sequence homology with the 'hect' family of E3 ligases, we proposed to identify substrates of hRPF1/Nedd4 ubiquitination activity with the goal of elucidating the mechanism for these observed PR and p53 transcriptional effects. Using a yeast two-hybrid approach, we have identified hPRTB (proline-rich protein, brain expressed) as a nuclear protein which interacts with and is a ubiquitination substrate of hRPF1/Nedd4 *in vitro* and in cultured cells. Furthermore, with the identification of a rev-like nuclear export sequence in hRPF1/Nedd4, we suggest that nuclear import/export of distinct 'hect' family members will contribute to the regulation of enzyme/substrate specificity within a cell. It is now apparent the potentiative effects of hRPF1/Nedd4 upon PR- and p53-dependent transcription are independent of ubiquitination activity of this enzyme. Nonetheless, the localization of hPRTB, a ubiquitination substrate of hRPF1/Nedd4, in splicing factor-rich nuclear speckles remains suggestive of a potential link between ubiquitination and the general transcriptional machinery.

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Table of Contents

Introduction

Our longstanding interest in cellular proteins and pathways which influence the transcriptional activity of the progesterone receptor led to the identification of yeast RSP5 and the its human homolog hRPF1/Nedd4 as modulators of progesterone receptor-dependent transcription [1]. Given the homology of hRSP5 and hRPF1/Nedd4 with the 'hect' family of E3 ubiquitin ligases, a class of enzymes implicated in the covalent attachment of ubiquitin to substrate molecules [2], we proposed initially that the ubiquitin-dependent degradation of a substate of hRPF1/Nedd4 might explain these observed effects upon PR-dependent and p53 dependent transcription. However, work from this and other laboratories [3], have demonstrated that the effects of hRPF1/Nedd4 upon transcription by steroid receptors is not dependent upon the ubiquitin ligase activity of hRPF1/Nedd4. Nonetheless, we have remained interested in the identification and characterization of novel substrates of the ubiquitination activity of hRPF1/Nedd4, and present within this summary report the description of one such substrate, human proline-rich transcript, brain expressed (hPRTB) [4].

Body

With this final summary, we report the accomplishment of each task outlined within the original Statement of Work. As indicated in previous progress reports, the preparation for the yeast two-hybrid assay (Task #1), screening of a HeLa cDNA library for interacting proteins (Task #2), and confirmation of true binding partners (Task #3) was all accomplished on schedule and led to our identification of six proteins which specifically interact with the amino terminus of hRPF1/Nedd4. Data representing the culmination of each of these tasks is summarized in the detailed description of hPRTB as a 'PPXY' motif-containing protein which directly interacts with the WW domains of hRPF1/Nedd4 [4].

Our efforts to purify recombinant full-length hRPF1/Nedd4 with enzymatic activity (Task #4) were initially frustrated; however, after utilizing either baculovirus/SF9 or bacterial expression systems, we ultimately were successful in producing an enzymatically active variant of hRPF1/Nedd4, aa. 190-900 (whect) [4]. This is consistent with reports of others that enzymatic activity of the full-length

hRPF1/Nedd4 protein has not yet been observed [5], and may suggest that proteoysis of hRPF1/Nedd4 contributes to the regulation of this ubiquitin ligase.

Utilizing recombinant hRPF1/Nedd4 (whect) enzyme, we assayed the ability of each hRPF1/Nedd4 binding partner to be ubiquitinated in vitro (Task #5). Each 'PPXY'-containing binding protein which interacted with the WW domains of hRPF1/Nedd4 was able to be ubiquitinated *in vitro*. Indeed, an intact 'PPXY' motif was requisite for hRPF1/Nedd4 ubiquitination of each candidate substrate, just as described for the interacting protein, hPRTB [4]. Two interacting proteins which associated with the amino terminal C2 domain of hRPF1/Nedd4 were not ubiquitination substrates, and are predicted to contribute to the regulation of activity or localization within a cell.

One particular substrate of hRPF1/Nedd4, hPRTB (proline-rich transcript, hPRTB), was further characterized for its ubiquitin-dependent degradation in a cellular environment (Task #6), as is described in detail in the second annual progress report as well as the appended manuscript [4]. However, our analysis of each of the additional *in vitro* ubiquitination substrates within cells failed to confirm the identity of these proteins as 'bona fide' substrates of hRPF1/Nedd4 activity. Possible explanations are that 1) the *in vitro* ubiquitination assay is promiscuous, in that proteins may be ubiquitinated in reticulocyte experiments which do not colocalize in a cell, or 2) such proteins may in fact be hRPF1/Nedd4 substrates, but we have not yet identified the proper hRPF1/Nedd4 activating stimulus which effects the ubiquitination of this subset of substrates. Lastly, the component of Task #6 which addresses the role of substrate proteins (such as hPRTB) in the transcriptional activation by the progesterone receptor and p53 was no longer feasible, given observations detailed in previous progress reports that the potentiative effect of hRPF1/Nedd4 in these assays is not dependent upon the catalytic activity of this ubiquitin ligase.

Though not proposed in the original Statement of Work, we have furthered our observations of hRPF1/Nedd4 action, in attempt to understand how an enzyme which is primarily cytoplasmic [6] might be able to target substrates within the nucleus. Our identification of a consensus rev-like nuclear export sequence within hRPF1/Nedd4 highlights a nuclear import/export mechanism as a crucial component of 'hect' ubiquitin ligase enzyme/substrate regulation [4].

Thus, all of the proposed tasks delineated in the original Statement of Work have been met. In addition to these described research accomplishments, other training opportunities have included a weekly student seminar series which afforded multiple opportunities for presentation of literature and research, and a Signal Transduction Colloquium series, after which students were weekly invited to share lunch and conversation with invited speakers. Regular lab meetings ongoing in the McDonnell lab consistently focused upon steroid receptor pharmacology, and discussion of the potential clinical implications for novel experimental results.

KEY RESEARCH ACCOMPLISHMENTS

•Completion of yeast two-hybrid screen with identification of 6 proteins which specifically bind to the amino terminal 'substrate binding domain' of hRPF1/Nedd4

•Confirmation of yeast two-hybrid interactions using GST-pulldown interaction assays

•Cloning of full-length cDNAs for two hRPF1/Nedd4 interacting proteins

•Colocalization of hPRTB with splicing machinery in nuclear speckles

•Creation of recombinant baculovirus for hRPF1/Nedd4 expression; purification of hRPF1/Nedd4 and hRPF1/Nedd4-C867A from baculovirus/SF9 cells

•Establishment of *in vitro* ubiquitination assay using yRSP5 or hRPF1/Nedd4 as E3 ubiquitin ligase

•Identification and creation of amino acid substitutions in substrate proteins which abrogate ubiquitination

•Isolation of *in vivo* ubiquitin conjugates of hPRTB within cells

•Pulse chase demonstration of degradation of hPRTB dependent upon intact 'PPXY' motif

•Observed increased degradation of hPRTB in presence of exogenous hRPF1/Nedd4 (but not catalytically inactive hRPF1/Nedd4-C867A)

•Deletion mapping and identification of a nuclear export sequence between aa. 297-307 of hRPF1/Nedd4

REPORTABLE OUTCOMES

PUBLICATIONS:

Maria Huacani Hamilton, Irina Tcherepanova, Jon M. Huibregtse, and Donald P. McDonnell. (2001) Nuclear Import/Export of hRPF1/Nedd4 Regulates the Ubiquitin-dependent Degradation of Its Nuclear Substrates. *Journal of Biological Chemistry* **276**:26324-26331.

Akihiro Ito, Chun-Hsiang Lai, Xuan Zhao, Shin'ichi Saito, **Maria H. Hamilton**, Ettore Appella and Tso-Pang Yao. (2001) p300/CBP-mediated p53 acetylation is commonly induced by p53-activating agents and inhibited by MDM2. *EMBO Journal* **20**:1331-1340.

Sylvie L. Beaudenon, **Maria R. Huacani**, Guangli Wang, Donald P. McDonnell, and Jon M. Huibregtse. (1999) The Rsp5 ubiquitin-protein ligase mediates DNA damage-induced degradation of the large subunit of RNA polymerase II in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **19**:6972-6979.

John D. Norris, Lisa A. Paige, Dale J. Christensen, Ching-yi Chang, **Maria R. Huacani**, Daju Fan, Paul T. Hamilton, Dana M. Fowlkes, Donald P. McDonnell. (1999) Peptide Antagonists of the Human Estrogen Receptor. *Science* **285**: 744-746.

CONFERENCE PRESENTATIONS AND POSTERS:

Maria R. Huacani and Donald P. McDonnell. PRTB, a novel proline-rich protein, is a substrate of the E3 Ubiquitin Ligase, hRPF1/Nedd4. Era of Hope Breast Cancer Meeting, Atlanta, Georgia (2000).

Maria R. Huacani and Donald P. McDonnell. Identification of a Novel Ubiquitination Substrate for the Nuclear Receptor Coregulator hRPF1/Nedd4. Keystone Symposia: Nuclear Receptors 2000, Steamboat Springs, Colorado (2000)

Maria R. Huacani, Sylvie L. Beaudenon, Jon M. Huibregtse, and Donald P. McDonnell. Identification of Substrates of hRPF1: A Novel E3 Ubiquitin Ligase. ISREC Conference: Cancer and the Cell Cycle, Lausanne, Switzerland (1999).

REPORTABLE OUTCOMES

DEGREES OBTAINED:

As a culmination of the research and training experiences delineated here, I successfully completed and defended my dissertation, graduating from Duke University with a Ph.D. in Biological Sciences in December of 2000.

EMPLOYMENT / FELLOWSHIPS RECEIVED:

I have been accepted by and currently work as a postdoctoral fellow in the laboratory of Dr. John D. Hildebrandt, Department of Pharmacology, at the Medical University of South Carolina. Additionally, I have applied for and been chosen after an interview process for two years of NIH funding support on an institutional postdoctoral training grant entitled "Training to Improve Cardiovascular Drug Therapy."

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Conclusions:

The proposed work has led to the identification of hPRTB as a novel nuclear substrate for the ubiquitin ligase hRPF1/Nedd4. We have additionally established a mechanism by which this primarily cytoplasmic ubiquitin ligase enzyme is able to target a nuclear substrate, with our discovery of a rev-like nuclear export sequence within hRPF1/Nedd4. However, we have also made the enigmatic observation that the potentiation of activated transcription by the progesterone receptor or p53 appears to be independent of the catalytic activity of hRPF1/Nedd4. Nonetheless, we do acknowledge that hRPF1/Nedd4 may indeed be able to impact general transcription events via a ubiquitination of a substrate such as hPRTB. Future studies exploring the possible role for the ubiquitination substrate hPRTB in RNA processing/splicing events have the potential to further such a link between ubiquitination and transcription.

Careful regulation of ubiquitin ligase/substrate recognition is crucial for cellular homeostasis, as several disease states have been identified to result from inappropriate (human papillomavirus degradation of p53) or decreased ability (mutations in sodium channel which block Nedd4 ubiquitination) to bind or ubiquitinate substrate proteins. These research conclusions add important information to our understanding of the specificity of substrate choice for the hRPF1/Nedd4, a member of the WWhect family of E3 ubiquitin ligases. Our work does confirm the observations of others that the WW domains of these enzymes mediate substrate binding. Perhaps more importantly though, our observation that hRPF1/Nedd4 undergoes nuclear import/export uncovers a novel form of regulation previously undiscovered for these enzymes. As other human WWhect enzymes similar to hRPF1/Nedd4 do exist, we predict that the localization of each of these enzymes will limit the pool of its potential substrates, thus contributing to their specificity.

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PRTB, A NOVEL PROLINE-RICH PROTEIN, IS A SUBSTRATE OF THE E3 UBIQUITIN LIGASE hRPF1/NEDD4

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Previously our laboratory has identified an E3 ubiquitin ligase, hRPF1/Nedd4, as a modulator of progesterone and glucocorticoid receptor transcriptional activity. hRPF1/Nedd4 belongs to the 'hect' (homology to E6-AP at the carboxy terminus) family of E3 ubiquitin ligases which are characterized by a conserved carboxy terminal catalytic domain. While other 'hect' E3 ubiquitin ligases, including E6-AP, have been described as coactivators of nuclear receptors, we have also observed that hRPF1/Nedd4 is a potentiator of p53-dependent transcription. hRPF1/Nedd4 is likely to have many diverse cellular targets; however, as it appears to modulate two transcription factors known to play a role in breast cancer, we are interested in the identification of substrates of hRPF1/Nedd4's enzymatic activity which may explain its transcriptional effects.

Using the amino terminal substrate binding domain of hRPF1/Nedd4, we have employed a yeast two-hybrid approach to identify proteins which bind to and and serve as substrates of hRPF1/Nedd4's ubiquitinating activity. One such protein, hPRTB, is a proline-rich nuclear protein which binds to the WW domains of hRPF1/Nedd4 via a consensus WW domain-binding 'PPXY' motif. Using an *in vitro* ubiquitination assay, we have demonstrated that hPRTB is ubiquitinated by yRSP5 or hRPF1/Nedd4. Ubiquitination is dependent upon substrate binding, as a 'PY' mutant which is unable to bind hRPF1/Nedd4 is also deficient in ubiquitination. Endogenous hRPF1/Nedd4 (or perhaps a similar WW-hect E3 ligase) is able to recognize hPRTB as an *in vivo* substrate, effecting ubiquitination and degradation of wild-type hPRTB but not the 'PY' mutant in HeLa cells. Immunolocalization studies demonstrate that hPRTB colocalizes with the splicing factor, SC35, in nuclear speckles, suggesting a role for this novel proline-rich protein in RNA processing.

Thus, in our efforts to understand the mechanism by which the E3 ubiquitin ligase hRPF1/Nedd4 modulates PR and p53-dependent transcription, we have identified a novel proline-rich protein, hPRTB, as a ubiquitination substrate for hRPF1/Nedd4. We are intrigued by the possibility that hPRTB may have a role in RNA processing, as RNA processing and transcription are increasingly understood to be coupled events. It is likely that our observations linking the ubiquitination pathway and general transcription machinery will further our understanding of the complexity of transcriptional regulation.

The U.S. Army Medical Research and Materiel Command under DAMD17-98-1-8072 supported this work.

Identification of a Novel Ubiquitination Substrate for the Nuclear Receptor Coregulator hRPF1/Nedd4

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In our search for proteins which modulate the nuclear receptor transcriptional response, our laboratory has previously identified the E3 ubiquitin ligase, hRPF1/Nedd4, as a modulator of progesterone and glucocorticoid receptor transcriptional activity. While additional 'hect' E3 ubiquitin ligases, including E6-AP, have been described as coactivators of nuclear receptors, the mechanism of their transcriptional effect is not well understood. E3 ubiquitin ligase proteins are the enzymes which determine substrate specificity in the ubiquitin-proteasome pathway. hRPF1/Nedd4 shares amino acid homology with the 'hect' family of E3 ubiquitin ligases, characterized by a conserved carboxy terminal catalytic domain. As little is known about the specific cellular substrates of hRPF1/Nedd4, we have sought to identify ubiquitination substrates with the goal of understanding these observations linking the ubiquitin-proteasome pathway and the general transcription machinery.

Homologs of hRPF1/Nedd4 bind and ubiquitinate several proteins from distinct cellular pathways, including RNA polymerase II and the sodium epithelial channel (ENaC). Using a two-hybrid approach, we have identified and characterized a novel hRPF1/Nedd4 substrate, human PRTB, a 17 kD proline-rich nuclear protein. hPRTB binds to the WW domains of hRPF1/Nedd4 via a consensus WW domain-binding 'PPXY' motif. We have demonstrated that hPRTB can be ubiquitinated by yRSP5 or hRPF1/Nedd4 *in vitro*. Ubiquitination is dependent upon substrate binding, as a mutant (PY), which is unable to bind to hRPF1/Nedd4, is also deficient in ubiquitination. Unlike the 'PY' mutant, wild-type hRPTB is rapidly ubiquitinated and degraded in HeLa cells. Current efforts are focused on analyzing *in vivo* interactions between hRPF1/Nedd4 and hPRTB with the goal of furthering our understanding of the complexity of E3 ligase/substrate recognition and its impact upon nuclear receptor signalling. [Supported by Department of Defense Predoctoral Fellowship, DAMD17-98-1-8072]

IDENTIFICATION OF SUBSTRATES OF hRPF1: A NOVEL E3 UBIQUITIN LIGASE

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The ubiquitin-proteasome pathway is responsible for the regulation of protein stability in a wide variety of cellular processes, including gene transcription, cell cycle progression and signal transduction. E3 ubiquitin ligase proteins are the components of this multi-enzyme cascade which are believed to be key players in the selection of ubiquitination substrates. Several examples of ubiquitination dysregulation and subsequent cellular transformation have been shown to occur. The defect in these systems has been shown to occur primarily at the level of E3 ubiquitin ligase-substrate recognition. Our laboratory is interested in an E3 ubiquitin ligase, hRPF1, which was originally identified as a modulator of steroid receptor transcriptional activity. The yeast homolog of hRPF1, RSP5 has been shown to bind to and ubiquitinate the large subunit of RNA polymerase II. We postulate that there may be additional hRPF1 substrates, the identification of which will help to explain these observations linking E3 ubiquitin ligase activity and the general transcriptional machinery. Using a yeast two-hybrid approach, we have identified a pre-mRNA cleavage factor which specifically binds to and serves as a substrate for hRPF1. As RNA processing is known to be coupled to transcription, we are intrigued by the possibility that components of the RNA processing machinery might be regulated by ubiquitination. *In vitro* analysis of the regions of hRPF1 required for binding, suggest that in addition to putative ubiquitination substrates, we have also identified a class of proteins which may play a regulatory role in hRPF1 activity. Present work is aimed at determining the physiological significance of these interactions.

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Nuclear Import/Export of hRPF1/Nedd4 Regulates the Ubiquitin-dependent Degradation of Its Nuclear Substrates*

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The ubiquitin-protein ligase (E3), hRPF1/Nedd4, is a component of the ubiquitin-proteasome pathway responsible for substrate recognition and specificity. Although previously characterized as a regulator of the stability of cytoplasmic proteins, hRPF1/Nedd4 has also been suggested to have a role in the nucleus. However, in light of the cytoplasmic localization of hRPF1/Nedd4, it is unclear whether *bona fide* nuclear substrates of hRPF1/Nedd4 exist, and if so, what mechanism may allow a cytoplasmic ubiquitin ligase to manifest nuclear activity. Our search for nuclear substrates led to the identification of the human proline-rich transcript, brain-expressed (hPRTB) protein, the ubiquitination and degradation of which is regulated by hRPF1/Nedd4. Interestingly, hPRTB colocalizes with the splicing factor SC35 in nuclear speckles. Finally, we demonstrate that hRPF1/Nedd4 is indeed capable of entering the nucleus; however, the presence of a functional Rev-like nuclear export sequence in hRPF1/Nedd4 ensures a predominant cytoplasmic localization. Cumulatively, these findings highlight a nuclear role for the ubiquitin ligase hRPF1/Nedd4 and underscore cytoplasmic/nuclear localization as an important regulatory component of hRPF1/Nedd4-substrate recognition.

The posttranslational modification of proteins by ubiquitination has been shown to play an important role in the regulation of cell cycle progression, signal transduction, and transcriptional events within the cell. Covalent attachment of the 76-amino acid polypeptide ubiquitin to a substrate protein is a catastrophic signal, targeting the substrate for rapid degradation (1, 2). The specific enzymes involved in this process, E1,[1] E2, and E3, have been studied in great detail (3). A human ubiquitin-activating enzyme (E1) is responsible for the ATP-dependent activation of the ubiquitin polypeptide. Activated ubiquitin is subsequently transferred to a downstream ubiquitin carrier protein (E2), and in many cases to a ubiquitin-protein isopeptide ligase (E3), which mediates the final transfer of activated ubiquitin to a substrate protein. Evidenced by the numerous examples of cellular dysregulation resulting from aberrant ubiquitination (4, 5), this ultimate enzyme-substrate recognition step is crucial for cellular homeostasis. Accordingly, there is of late a heightened level of interest in defining the mechanisms that govern the target specificity of the various E3 ligases and how this event is regulated in target cells.

The experiments that have formed the foundation of our understanding of the role of E3 ligases were those that describe E6-associated protein and its ability to cooperate with the viral E6 protein in ubiquitinating p53 following human papillomavirus infection (6). This work led to the discovery of a family of proteins with sequence homology to E6-associated protein, the **h**omology to **E**6-associated protein at the **c**arboxyl **t**erminus (hect) family of proteins (7), and the observation that the amino terminus is the primary determinant of target specificity. Recent work demonstrating RNA polymerase II ubiquitination by yeast RSP5, Smad ubiquitination by Smurf1, and Notch ubiquitination by Itch confirm the importance of the amino terminus in target selection (8–10).

Nedd4 is a hect E3 ubiquitin ligase enzyme the transcript of which was originally identified to be developmentally down-regulated in neural precursor cells (11). The domain structure of hRPF1/Nedd4 places this E3 ligase in the WWhect subclass of ubiquitin ligases. This subclass of enzymes is characterized by 2–4 copies of a WW protein-protein interaction domain (12), followed by a conserved hect domain. The hect domain is the catalytic domain responsible for ubiquitination, an activity that is absolutely dependent upon an invariant cysteine residue located within the active site (7). Similar to many other human WWhect E3 ligases, hRPF1/Nedd4 contains a C2/CaLB domain at the amino terminus, which is responsible for mediating membrane localization in response to calcium (13).

Although hRPF1/Nedd4 contains a hect domain, implying that it is involved in ubiquitination, many studies performed thus far with putative targets have not been illuminating with respect to the function of the enzyme or how the activity is regulated. To date, the best characterized substrate of Nedd4 is the rat sodium epithelial channel (14–16). These studies, which demonstrate that Nedd4 can interact with and regulate the turnover of the sodium epithelial channel, imply a non-nuclear function of this enzyme. This contention is supported by additional work that indicates that Nedd4 and the cytoplasmic adapter protein mGrb10 interact (17). However, recent studies from our laboratories have demonstrated that hRPF1/

* This work was supported by a United States Army Research and Material Command Predoctoral Fellowship DAMD17-98-1-8072 (to M. H. H.) and by National Institutes of Health Grant DK50494 (to D. P. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Duke University Medical Center, Dept. of Pharmacology and Cancer Biology, Box 3813, Durham, NC 27710. Tel.: 919-684-6035; Fax: 919-681-7139; E-mail: mcdon016@acpub.duke.edu.

[1] The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase; aa, amino acids; EGFP, enhanced green fluorescent protein; GST, glutathione *S*-transferase; hect, homology to E6-associated protein at the carboxyl terminus; mut, mutant; NES, nuclear export sequence; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PRTB, proline-rich transcript, brain-expressed; hPRTB, human PRTB.

Nedd4 may have roles in the nucleus. Specifically, it has been shown that 1) a Nedd4 homolog, yeast RSP5, is responsible for the ubiquitination of the large subunit of RNA polymerase II in response to DNA damage (18), and 2) overexpression of hRPF1/Nedd4 alters the transcriptional activity of the progesterone receptor (19). In addition, an erythroid-specific transcription factor, NF-E2, has been shown to physically associate with hRPF1/Nedd4 (20), although the significance of this interaction remains to be determined. Cumulatively, these data suggest that hRPF1/Nedd4 can modulate target protein ubiquitination in both the cytoplasm and the nucleus. However, because hRPF1/Nedd4 is localized predominantly in the cytoplasm (21), the physiological significance of the interaction between hRPF1/Nedd4 and nuclear proteins is unclear. In an effort to evaluate the potential roles of hRPF1/Nedd4 in the nucleus and how these activities are manifest, we have undertaken a strategy to identify additional nuclear substrates of hRPF1/Nedd4 with a view to (*a*) confirming that this enzyme can interact with and regulate the stability of nuclear proteins and (*b*) defining a mechanism by which this cytoplasmic ubiquitin ligase can exert its activity in the nucleus.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Splicing factor SC35 monoclonal antibody was purchased from Sigma. Anti-c-Myc monoclonal antibody (9E10) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All horseradish peroxidase-conjugated secondary antibodies and ECL reagents were obtained from Amersham Pharmacia Biotech. Anti-mouse Texas Red-conjugated secondary antibodies, horse serum, and VectaShield with DAPI (4′,6-diamidino-2-phenylindole) were purchased from Vector Laboratories (Burlingame, CA).

Plasmids—The complete 5′ coding sequence of hRPF1/Nedd4 (aa 1–900) was obtained using 5′ rapid amplification of cDNA ends, polymerase chain reaction-amplified, and subcloned into an incomplete RPF1/Nedd4 cDNA (derived from pBKC-hRPF1) (19) using standard subcloning procedures. hRPF1/Nedd4 deletion constructs encoding C2 (aa 1–192), WW (aa 173–564), hect (aa 507–900), aa 293–900, aa 309–900, and aa 404–900 were subcloned by polymerase chain reaction from full-length hRPF1/Nedd4 constructs, and all sequences were verified by sequence analysis. To create the hRPF1/Nedd4-C867A mutant, we utilized the *Sac*I site just upstream of amino acid 867 for construct preparation. Briefly, a primer was designed (5′-gccaagagctcataccgcttttaatcgcc-3′) that allowed polymerase chain reaction amplification of amino acids 862–900, resulting in the incorporation of a two-base substitution that changed cysteine 867 to alanine. The *Sac*I-*Not*I fragment containing the C867A amino acid change was incorporated into the context of the full-length hRPF1/Nedd4 using standard subcloning procedures. Site-directed mutagenesis was used to introduce the PY mutation in hPRTB (P40A/Y42A), and the mutant nuclear export sequence (NES) (L307A/I309A) in hRPF1/Nedd4-C867A. Primers used were as follows: PRTB-PYmut, 5′-ccgatgctccagctgccgcctcagagctc-3′(sense) and 5′-gagctctgaggcggcagctggagcatcgg-3′ (antisense); hRPF1/Nedd4-C-867A-mutNES, 5′-gaattgaatgccagagccaccgcttttggaaattcagccg-3′ (sense) and 5′-cggctgaatttccaaaagcggtggctctggcattcaattc-3′ (antisense). The integrity of all constructs was verified by sequencing.

Enhanced green fluorescent protein (EGFP) fusion constructs were the result of subcloning hPRTB (from the library vector pGADGH) into the *Eco*RI-*Bam*HI sites of pEGFP-C1 (CLONTECH). To create Myc-tagged fusions, we inserted a *Bam*HI-*Bam*HI fragment of hPRTB cDNA (containing extra 5′ *Bam*HI-*Eco*RI linker sequence: ggatccccgaattc) into the *Bam*HI site of pcDNA3–5×Myc vector.

Cell Culture and Transfections—HeLa cells were cultured in minimal essential medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate and maintained in a humidified incubator at 37 °C, 5% CO_2. Cells were transiently transfected using Lipofectin (Life Technologies, Inc.) for 4 h and allowed to recover for 24–48 h prior to harvest and analysis.

Yeast Two Hybrid Screen—A cDNA encoding the amino terminus of hRPF1/Nedd4 (NW, aa 26–506) was subcloned into the vector pGBT9 (CLONTECH) and introduced by standard lithium acetate protocol into HF7c cells (CLONTECH). A HeLa Matchmaker library was sequentially transformed, and colonies were grown on selective plates containing 1 mM 3-aminotriazole. A HeLa cervical carcinoma cDNA library was chosen based upon previous observations (19) and immunodetection of endogenous hRPF1/Nedd4 protein product in HeLa cells. His+ clones were isolated after 10 days of growth at 30 °C, restreaked onto selective plates (1 mM 3-aminotriazole), and grown for 3 days prior to β-galactosidase filter lift assays. Library and bait plasmids were subsequently cotransformed into Y190 yeast cells to verify phenotype and to quantitate interaction using a liquid β-galactosidase assay. Briefly, a midlogarithmic phase culture (A_{600} = 0.5–0.8) was pelleted, washed, and subjected to two cycles of freeze-thaw lysis. The β-galactosidase activity of the lysate was measured at A_{578} and quantitated using CPRG (chlorophenol red-β-D-galactopyranoside) as a substrate.

Glutathione S-Transferase (GST)-Pulldown Interaction Assays—hPRTB was subcloned into pcDNA3 (Invitrogen), *in vitro* transcribed/translated, and radiolabeled in a rabbit reticulocyte lysate system (TNT, Promega). hRPF1/Nedd4 or deletions thereof (C2, aa 1–192; WW, aa 173–564; hect, aa 507–900) were fused to GST, expressed, and purified from bacteria. Purified GST fusion proteins were bound to glutathione-Sepharose (Amersham Pharmacia Biotech) and incubated with radiolabeled hPRTB in NETN-A (25 mM Tris 8.0, 75 mM NaCl, 0.1% Nonidet P-40, 2 mM EDTA) overnight at 4 °C. Bound proteins were washed in NETN-B (150 mM NaCl), eluted, and analyzed by SDS-PAGE followed by autoradiography.

In Vitro Ubiquitination Assays—Assays were performed essentially as described previously (18). Briefly, an *in vitro* translated, radiolabeled substrate was incubated with BL21 bacterial extracts overexpressing E1 and an E2 (UbcH5B). Reaction mixtures contained 25 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1 mM dithiothreitol, 2 mM ATP, 2 mM $MgCl_2$, 2 μg of bovine ubiquitin (Sigma), and 500 ng of purified E3 enzyme (either yRSP5 or hRPF1/Nedd4-whect). In some cases, an ATP regenerating system was also included, consisting of 0.1 M phosphocreatine, 3.5 units/ml creatine phosphokinase, and 0.6 units/ml inorganic pyrophosphatase. After incubation at 30 °C for 1 h, reactions were terminated with 3× SDS-PAGE sample buffer, resolved, and detected by SDS-PAGE followed by autoradiography.

Detection of in Vivo Ubiquitin Conjugates—Following a previously published procedure (15), HeLa cells were transiently transfected with mammalian expression plasmids for His-tagged ubiquitin and a Myc-tagged substrate (hPRTB or PY mutant). Thirty-six hours after transfection, cells were harvested in lysis buffer (PBS, 1% Triton X-100, 10% glycerol). The insoluble fraction was removed by a high speed spin, and the clarified supernatant was denatured by addition of 2% SDS followed by boiling for 5 min. Denatured extract was diluted with 12 volumes of lysis buffer and incubated with 50 μl of nickel-nitrilotriacetic acid resin (Qiagen) for 4 h at 4 °C. After thorough washing with lysis buffer containing 300 mM NaCl and 40 mM imidazole, His-tagged proteins were eluted with 3× SDS sample buffer and analyzed by Western immunoblot analysis using an anti-c-Myc antibody, 9E10.

Pulse-Chase Analysis—HeLa cells (~70–80% confluency) that had been plated in 60 mM dishes were transiently transfected with 2.7 μg of a Myc-hPRTB or Myc-hPRTB-PYmut expression plasmid and 20 ng of the internal control Myc-EGFP. In experiments assaying the effect of exogenous hRPF1/Nedd4 on PRTB stability, 1.3 μg of pc:RPF1 or pc:RPF1-C867A (or an equimolar amount of empty pcDNA3 vector) was cotransfected as well. Twenty-four hours posttransfection, cells were washed with PBS and incubated in methionine- and cysteine-free medium for 30 min. A radioactive mixture of methionine and cysteine (175 μCi of Tran^{35}S-Label, ICN) was used to metabolically label cells for 2 h, after which the medium was removed and replaced with cold medium containing an excess of methionine and cysteine (3 and 1 mM, respectively) to chase for indicated time points. At each time point, cells were harvested in PBS, 0.5% Triton X-100 plus protease inhibitors, flash-frozen, and subsequently immunoprecipitated with an antibody directed against the Myc tag (9E10, Santa Cruz Biotechnology). Immunoprecipitates were analyzed by SDS-PAGE and autoradiography and quantitated using a phosphorimager.

Indirect Immunolocalization—HeLa cells were transiently transfected with an expression plasmid for an EGFP-hPRTB (or PY mutant) fusion protein and plated onto 25-mm round glass coverslips. Cells were fixed using 4% paraformaldehyde for 10 min and permeabilized in 0.5% Triton X-100 for 10 min. Samples were blocked in PBS containing 10% horse serum and then incubated with anti-SC35 (Sigma) at a dilution of 1:2000 in PBS/2% horse serum, followed by incubation with anti-mouse Texas Red-conjugated secondary antibody at a 1:75 dilution in PBS/2% horse serum. Coverslips were mounted in VectaShield plus DAPI (4′,6-diamidino-2-phenylindole). Localization of transfected Myc-hRPF1/Nedd4 deletion or mutation constructs was performed essentially as described above, only using the anti-c-Myc antibody (9E10) at a 1:2000 dilution. Indicated samples were treated with 20 ng/ml of leptomycin B

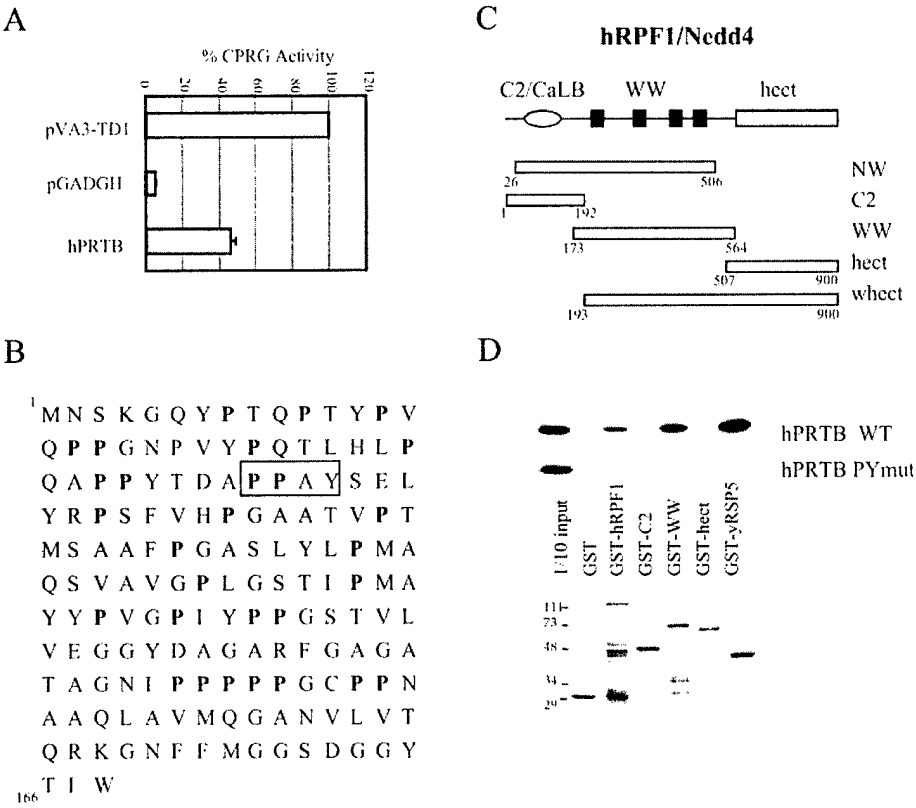

FIG. 1. **hRPF1/Nedd4 Interacts (via its WW domains) with the PPAY motif of hPRTB.** *A*, yeast two hybrid interaction between aa 26–506 of hRPF1/Nedd4 and hPRTB. Yeast Y190 cells were cotransformed with plasmids encoding Gal4DBD-NW and hPRTB-AD. Midlogarithmic cultures were analyzed in triplicate for LacZ activity using chlorophenol red-β-D-galactopyranoside (CPRG) as a substrate. Data are represented as a percentage of the positive control interaction, pVA3-TD1 (p53 and SV40-T antigen). *B*, protein sequence for human PRTB. The PPAY motif, which was mutated in this study, is indicated by the *outlined rectangle*. The numerous proline residues are highlighted by *boldface type*. *C*, hRPF1/Nedd4 deletion constructs encoding NW (aa 26–506), C2 (aa 1–192), WW (aa 173–564), hect (aa 507–900), and whect (aa 193–900). *D*, GST-pulldown interactions between GST-hRPF1/Nedd4 and Myc-hPRTB. GST alone, GST fusions of hRPF1/Nedd4 (or indicated portions), or GST-yRSP5 was immobilized on glutathione-Sepharose and incubated with ^{35}S-labeled Myc-PRTB or Myc-PRTB-PYmut. Bead-bound proteins were analyzed by SDS-PAGE and autoradiography. Fusion proteins used for GST-pulldown interaction studies are shown in the *lower panel*. Approximately equivalent microgram amounts of GST or GST fusion proteins were resolved by SDS-PAGE and detected by Coomassie Blue staining. *WT*, wild type.

for 4 h prior to fixation and sample preparation. A Zeiss LSM410 laser scanning confocal microscope with a krypton/argon laser (Carl Zeiss Inc., Thornwood, NY) was used for confocal microscopy.

RESULTS

Identification of Potential Substrates for hRPF1/Nedd4 Using a Yeast Two Hybrid Screen—Several nuclear proteins have been shown to be ubiquitinated by hRPF1/Nedd4 *in vitro*; however, a validated target of this enzyme in the nucleus has not been established in intact mammalian cells. With the goal of identifying *bona fide* cellular substrates of the catalytic activity of hRPF1/Nedd4, we used the amino terminus of hRPF1/Nedd4 in a yeast two hybrid screen to identify novel binding partners, a subset of which we would predict to be ubiquitination substrates.

As a result of our screen, six cDNAs were isolated, all encoding a 17-kDa proline-rich protein, which specifically interact with aa 26–506 of hRPF1/Nedd4 (Fig. 1*A*). Homology searches using BLAST programs indicated that this 17-kDa protein is identical to KIAA0058, a cDNA isolated from a myeloid cell line, KG-1. We and others have termed this human cDNA, hPRTB (Fig. 1*B*), based upon its high amino acid identity with mouse proline-rich transcript, brain-expressed (PRTB) protein, which was isolated in a gene trap screen as a transcript expressed in the developing mouse inner ear (22). Although its amino acid sequence does not share significant sequence homology with that of other proteins, the most notable feature of hPRTB is its proline-rich composition (18%).

To independently verify that hPRTB does indeed interact with full-length hRPF1/Nedd4, we assayed the ability of *in vitro* translated, [^{35}S]methionine-labeled Myc-hPRTB to interact with recombinant GST fusions of hRPF1/Nedd4 (see schematic in Fig. 1*C*). Myc-hPRTB was able to interact with full-length hRPF1/Nedd4 or yRSP5, a yeast homolog that has a domain structure similar to that of hRPF1 (containing only three WW domains). The WW domains of hRPF1/Nedd4 were sufficient for interaction with hPRTB; it is important to note that the greater hPRTB signal associated with GST-WW (Fig. 1*D*) is likely a reflection of the greater molar amount of GST-WW used. Neither the hect domain nor the C2 domain resulted in any detectable interaction (Fig. 1*D*). WW domains are predicted to interact with proline-containing consensus sequences, which are either PPXY, PPLP, or PGM (12, 23–25). Examination of the proline-rich regions within hPRTB revealed a consensus PPAY motif located in the central portion of the protein (Fig. 1*B*). It is interesting to note that the WWhect ligase, Smurf1, similarly binds to a PPAY motif in its substrate, Smad1 (9). With the prediction that this conserved motif may mediate the interaction of hPRTB with hRPF1/Nedd4, we substituted the second proline and subsequent tyrosine with alanine and assayed for the ability of this PY mutant to interact with hRPF1/Nedd4. The two-amino acid substitution within the PPAY motif in hPRTB was able to disrupt the ability of hPRTB to bind to hRPF1/Nedd4 (Fig. 1*D*), further demonstrating that the WW domains of hRPF1/Nedd4 directly interact with the PPAY motif of hPRTB. The direct association of a WW domain-containing enzyme and a substrate with a PPXY motif is ideal for an enzyme-substrate interaction in that WW domains typically bind with high specificity rather than high affinity (26), a property that may explain our inability to isolate hRPF1/Nedd4-hPRTB complexes from cellular extracts (data not shown).

hPRTB Colocalizes with Splicing Factors in Nuclear Speckles—Given our interest in a possible nuclear function of hRPF1/Nedd4, we next sought to determine the subcellular localization of the potential ubiquitination substrate hPRTB. To this end, we fused hPRTB to EGFP and analyzed its subcellular localization using fluorescence confocal microscopy. EGFP-hPRTB and the corresponding PY mutant (P40A/Y42A) have identical fluorescence patterns and are localized to the nucleus in a discrete speckled pattern (Fig. 2). We were intrigued by our observation of the localization of hPRTB to spots in the nucleus, reminiscent of "nuclear speckles," which are enriched in

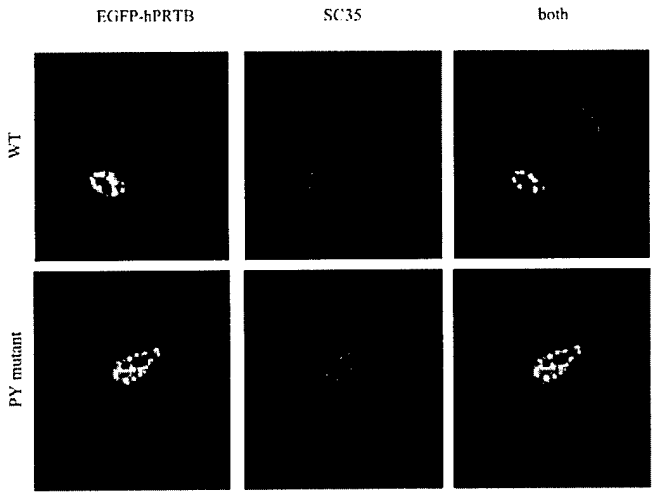

FIG. 2. **hPRTB colocalizes with splicing factor SC35 in nuclear speckles.** The EGFP-N1 vector was used to express hPRTB as a green fluorescent protein fusion protein in HeLa cells. Twenty-four hours posttransfection, cells were plated onto glass coverslips, allowed to attach, and subsequently fixed, permeabilized, and incubated with a monoclonal antibody that recognizes the nuclear speckle-associated splicing factor SC35 (27). Green fluorescent protein or mouse-Texas Red fluorescence was detected using fluorescence confocal microscopy.

splicing factors and contain a population of hyperphosphorylated RNA polymerase II (27, 28). Using fluorescent confocal microscopy, we demonstrated that both hPRTB and the PY mutant indeed colocalize with the splicing factor, SC35, in nuclear speckles (Fig. 2), suggesting that hPRTB may have a role in the transcription and/or splicing of RNA transcripts. Thus, in addition to identifying a potential nuclear substrate of hRPF1/Nedd4, we have localized hPRTB to splicing factor-rich speckles, a subnuclear localization where a population of RNA polymerase II, another WWhect E3 substrate, is known to reside.

hPRTB Is a Substrate of WW Hect E3 Ubiquitin Ligases in Vitro—Given that hPRTB specifically binds to hRPF1/Nedd4, we next wanted to determine whether it could serve as a substrate for the E3 ubiquitin ligase activity of this enzyme. A recombinant hRPF1/Nedd4 (whect) derivative lacking the amino-terminal C2 domain was used in enzymatic assays, as full-length protein is not active under the conditions tested (18). Efficient multi-ubiquitination of hPRTB was observed when assayed in the presence of purified hRPF1/Nedd4 (whect) or yRSP5, but not the hect E3 ligase E6-AP, which lacks WW domains in its amino terminus (Fig. 3*A*). Additionally, no ubiquitination was observed when either the E2 (UbcH5B) or E3 (RPF1/Nedd4-whect) enzyme was omitted from the reaction mixture (data not shown and Fig. 3*A*). With the prediction that substrate binding is necessary for E3 ligase activity, we next tested the hypothesis that the hPRTB-PYmut would not be ubiquitinated in this assay. Indeed, mutation of two key residues within the PPAY motif of hPRTB was able to completely abrogate ubiquitination of hPRTB by either hRPF1/Nedd4 (whect) (Fig. 3*B*) or yeast RSP5 (data not shown). Thus, a strong correlation between binding of hPRTB to hRPF1/Nedd4 and its ability to be ubiquitinated *in vitro* was established.

hPRTB Requires an Intact PPXY Motif to Support Ubiquitin Conjugation and Degradation in Cells—Having confirmed that hPRTB is an efficient substrate for Nedd4 *in vitro*, we next turned to evaluating whether hPRTB is a physiological substrate for ubiquitination. Specifically, we transfected HeLa cells with plasmids expressing a His-tagged ubiquitin protein and Myc-tagged hPRTB. In theory, ubiquitination substrates should be modified by His-ubiquitin, and the resultant conjugates can be isolated on a nickel-nitrilotriacetic acid resin. As indicated in Fig. 3*C, lanes 4–6,* His-ubiquitin conjugates were detected upon cotransfection of His-ubiquitin and wild type Myc-hPRTB. The migration of these 40–60-kDa ubiquitin conjugates differs from the slower migrating multi-ubiquitinated conjugates observed *in vitro* (Fig. 3, *A* and *B*), perhaps due to the regulated activity of an endogenous (rather than purified) E3 enzyme, coupled with the efficient cellular degradation of multi-ubiquitinated species within cells. Transfection of either Myc-hPRTB alone (Fig. 3*C, lane 4*) or cotransfection of Myc-hPRTB-PYmut and His-ubiquitin (*lane 6*) was unable to produce His-ubiquitin hPRTB conjugates. Western analysis (Fig. 3*C, lanes 1–3*) was used to verify that all proteins were expressed in HeLa cellular lysates. It was observed that Myc-hPRTB-PYmut accumulates to a higher steady state level than Myc-PRTB (Fig. 3*C,* compare *lane 3* with *lanes 1* and *2*), a finding that led us to consider that the stability of wild type and PY mutant hPRTB proteins may be different. Consequently, we analyzed the half-lives of Myc-hPRTB and Myc-hPRTB-PYmut in HeLa cells using pulse-chase analysis. As shown in Fig. 4*A,* wild type hPRTB has a significantly shorter half-life than the hPRTB-PYmut, the mutant that is unable to support ubiquitination. Averaging the data from several independent experiments, we conclude that mutation of two key residues within the PPAY motif of hPRTB results in a 3.5-fold increase in the half-life of hPRTB (2 h to 7 h) (Fig. 4*B*). Cumulatively, these observations provide compelling evidence that hPRTB is a physiological substrate of an endogenous WW-hect E3 ubiquitin ligase, such as hRPF1/Nedd4.

hRPF1 Regulates the Stability of hPRTB in Cells—It has previously been shown that nuclear proteins such as the large subunit of RNA polymerase II and the transcription factor NF-E2 bind to or are able to be ubiquitinated by mammalian Nedd4 family members *in vitro* (18, 20, 29); however, as of yet, there is no evidence that such proteins are physiologically regulated by a particular mammalian E3 ubiquitin ligase within cells. Given our data thus far, we suspected that the WW-hect E3 ubiquitin ligase responsible for the ubiquitin-dependent degradation of PRTB was hRPF1/Nedd4. Thus, we sought to establish that exogenous hRPF1/Nedd4 was able to alter the ubiquitin-dependent degradation of hPRTB. Using pulse-chase analysis, we demonstrated that the degradation of PRTB was accelerated in samples containing transiently transfected hRPF1/Nedd4 but not in samples transfected with the catalytic mutant hRPF1/Nedd4-C867A or an empty expression plasmid (Fig. 5*A*). Specifically, the half-life of PRTB in the presence of overexpressed hRPF1/Nedd4 was 70 min, compared with a $t_{1/2}$ of 150 min in samples containing either hRPF1/Nedd4-C867A or no exogenous hRPF1/Nedd4 (Fig. 5*B*). Western blot analysis confirmed that the total amount of hRPF1/Nedd4 or hRPF1/Nedd4-C867A protein in transfected cells was at least 2–3 times the amount normally present within HeLa cells (Fig. 5*C*). Thus, we have identified a nuclear speckle-associated protein, hPRTB, as a substrate of the E3 WW-hect ubiquitin ligase hRPF1/Nedd4 within cells. These data provide direct evidence that hRPF1/Nedd4 can interact with, ubiquitinate, and regulate the stability of a confirmed nuclear protein in intact cells.

hRPF1/Nedd4 Contains a Rev-like Nuclear Export Sequence—To further substantiate our observations that hRPF1/Nedd4 is able to target the nuclear protein hPRTB for ubiquitination and degradation, we finally sought to understand the mechanism by which a primarily cytoplasmic E3 enzyme, hRPF1/Nedd4, is able to modify the nuclear protein hPRTB.

hRPF1/Nedd4 has been reported to contain a bipartite nuclear localization signal between amino acids 534–550; how-

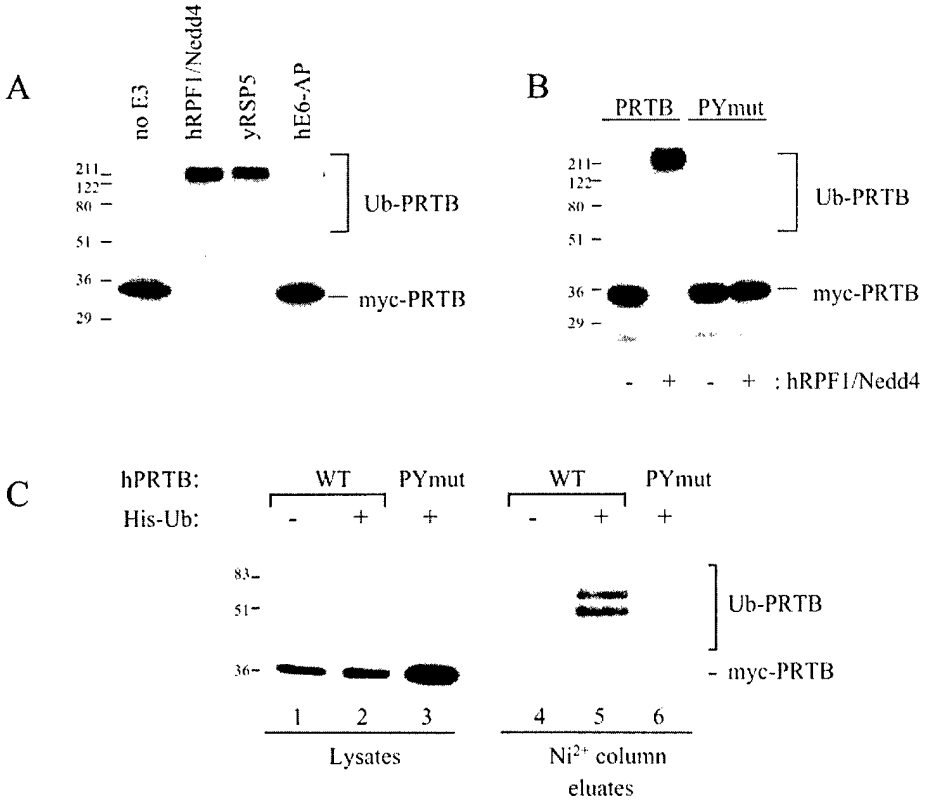

FIG. 3. **hPRTB is an *in vitro* and *in vivo* ubiquitination substrate.** *A*, hRPF1/Nedd4 ubiquitinates hPRTB *in vitro*. ^{35}S-Labeled Myc-hPRTB was incubated with purified hRPF1/Nedd4 (whect), yRSP5, or hE6-AP in the presence of ATP, ubiquitin, and bacterially expressed E1 and E2 (UbcH5B) enzymes. *B*, the hPRTB-PY mutant is unable to be ubiquitinated by hRPF1/Nedd4 (whect). hPRTB and hPRTB-PYmut, containing a two-amino acid substitution (P40A/Y42A) of the PPAY motif, were assayed in a standard ubiquitination assay using E1, E2 (UbcH5B), and hRPF1/Nedd4 (whect) as the E3 enzyme. *C*, His-ubiquitin conjugates of hPRTB isolated from HeLa cells. HeLa cells were transfected with expression plasmids for His-ubiquitin and Myc-hPRTB or Myc-hPRTB-PYmut. Forty hours posttransfection, denatured lysates were prepared (*lanes 1–3*), and His-conjugates were purified on nickel resin (*lanes 4–6*). Myc-hPRTB or Myc-hPRTB His-ubiquitin conjugates were detected by Western immunoblot analysis using an antibody directed against c-Myc (9E10). *WT*, wild type.

ever, it has been suggested in the past that Nedd4 is primarily a cytoplasmic protein (21, 30). Indeed, when Myc-tagged RPF1 is expressed in HeLa or NIH3T3 cells, we have shown that it is primarily cytoplasmic (data not shown). In an attempt to artificially place hRPF1/Nedd4 into the nuclear compartment of cells, we fused a strong SV40 nuclear localization signal to the amino terminus of hRPF1/Nedd4. When cellular localization of SV40 nuclear localization signal-RPF1/Nedd4 was assayed, little to no nuclear staining was detected despite significant cytoplasmic staining (data not shown), raising the possibility that hRPF1/Nedd4 is a protein that is constitutively exported from the nucleus.

Many nuclear proteins undergo nuclear export via the CRM1-dependent nuclear export pathway (31–33). To determine whether the WWhect E3 ligase hRPF1/Nedd4 might be a substrate of this nuclear export system, we evaluated hRPF1/Nedd4 localization in the presence of the drug leptomycin B, a specific inhibitor of CRM-1 dependent export (34). Myc-hRPF1/Nedd4-expressing cells or Myc-hRPF1/Nedd4-C867A-expressing cells were treated with 20 ng/ml leptomycin B, and the subcellular localization of hRPF1/Nedd4 was analyzed by immunofluorescence with an antibody directed against the Myc tag. The results of this analysis (Fig. 6*A*) indicate that a population of both wild type and catalytically inactive hRPF1/Nedd4 were localized within the nucleus after treatment with leptomycin B, indicating that this WWhect E3 ubiquitin ligase, or a complex containing this protein, is a substrate of CRM-1 dependent nuclear export.

Given the data of others that a hRPF1/Nedd4 derivative encoding aa 404–900 is localized primarily within the nucleus (21), whereas full-length constructs are cytoplasmic, we next used a series of amino-terminal deletion constructs to map the region of hRPF1/Nedd4 responsible for its cytoplasmic localization. Full-length hRPF1/Nedd4, as well as aa 293–900, localized primarily to the cytoplasm, but proteins encoding aa 309–900 and 404–900 were present in both the cytoplasm and the nucleus (data not shown), suggesting that a sequence between amino acids 293 and 309 of hRPF1/Nedd4 is responsible for its steady state cytoplasmic localization. Therefore, we compared amino acids 293–309 of hRPF1/Nedd4 with the leucine-rich consensus for Rev-like nuclear export. Indeed, amino acids 297–307 of hRPF1/Nedd4 contain sequence identity with this NES consensus and share significant homology with the NESs found in other proteins, such as PKI, HIVrev, human p53, and Rex (Fig. 6*B*) (31, 35–37). To prove that this sequence within hRPF1/Nedd4 is able to act as an NES, we substituted conserved residues with alanine (L305A and I307A) and assayed for the cellular localization of this putative NES mutant. Mutation of these two conserved amino acids within the export sequence significantly increased the amount of hRPF1/Nedd4 protein that was detected in the nucleus, as compared with the primarily cytoplasmic localization of Myc-hRPF1/Nedd4-C867A (Fig. 6*C*) or Myc-RPF1 (data not shown). Interestingly, when the NES mutant was created in the context of a wild type hRPF1/Nedd4, extremely low quantities of exogenous protein were detected by Western blot and immunofluorescence analysis. Nonetheless, these observations cumulatively demonstrate that amino acids 297–307 mediate the CRM1-dependent nuclear export of hRPF1/Nedd4.

DISCUSSION

Presented in this study is evidence that hPRTB, a proline-rich protein that colocalizes with splicing machinery in nuclear speckles, is a *bona fide* nuclear substrate of the WW hect E3 ubiquitin ligase, hRPF1/Nedd4. This identification of a nuclear substrate extends the role of the hRPF1/Nedd4 ubiquitin ligase to nuclear proteins. Furthermore, deletion and mutational analyses have led to our subsequent identification of a leucine-rich rev-like nuclear export sequence within hRPF1/Nedd4. Thus, we propose that nuclear import/export is an important component of the regulation between the primarily cytoplasmic E3 enzyme, hRPF1/Nedd4, and its nuclear substrate, hPRTB.

An in Vivo Nuclear Substrate of hRPF1/Nedd4—It has been well established that Nedd4 can interact with and modulate

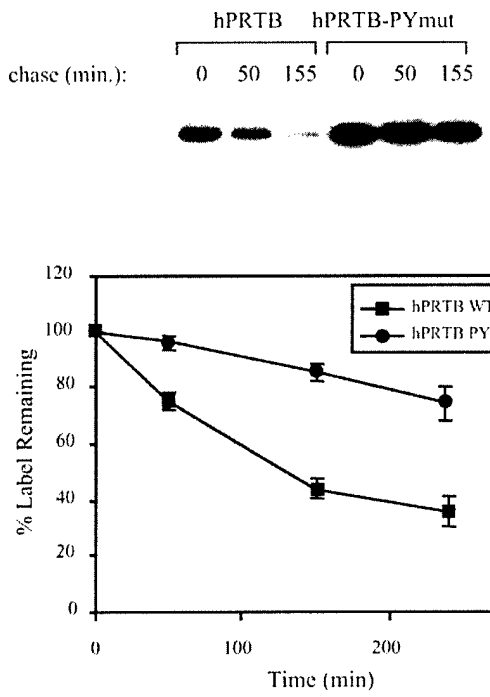

FIG. 4. **Mutation of the PPAY motif in hPRTB prolongs its half-life.** *A*, pulse-chase analysis of hPRTB and its PY mutant in HeLa cells. HeLa cells were transfected with Myc-hPRTB or Myc-hPRTB-PY mut, metabolically labeled, and chased in cold medium. Lysates from indicated time points were immunoprecipitated using a c-Myc antibody (9E10) and analyzed by SDS-PAGE followed by autoradiography. *B*, half-lives of wild type and PY mutant of hPRTB. Results from four independent pulse-chase experiments were quantitated using a phosphorimager, normalized against an internal Myc-EGFP control, and expressed as percentage of labeled protein at time 0.

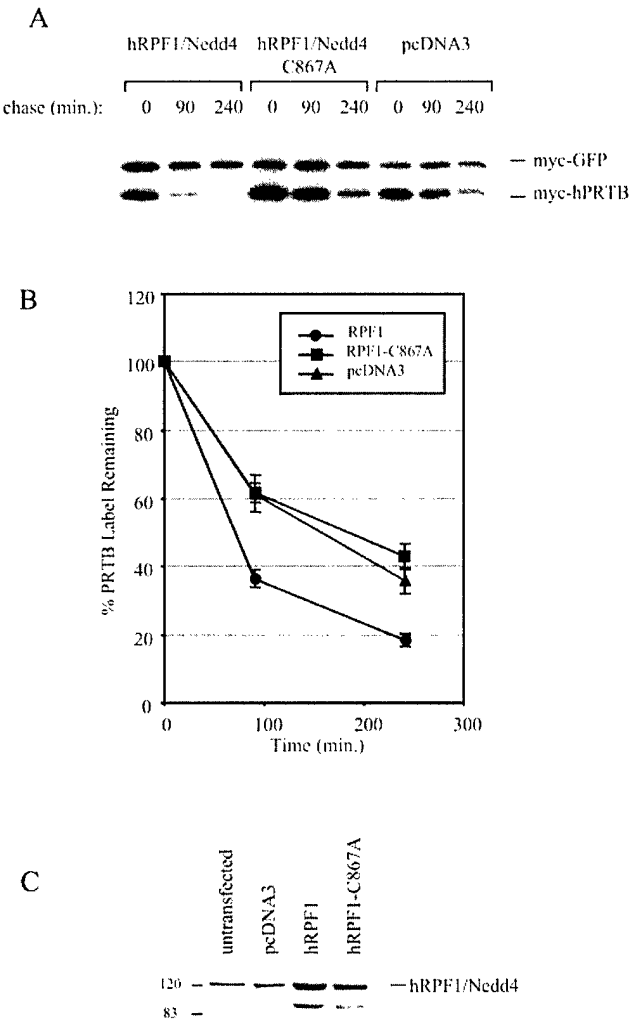

FIG. 5. **hRPF1/Nedd4, but not its C867A catalytic point mutant, accelerates degradation of hPRTB.** *A*, HeLa cells were cotransfected with Myc-PRTB, the internal control Myc-EGFP, and pcDNA3, hRPF1/Nedd4, or hRPF1/Nedd4-C867A. Cells were subsequently radiolabeled and chased in cold medium. Lysates from indicated time points were immunoprecipitated using a c-Myc antibody (9E10) and analyzed by SDS-PAGE followed by autoradiography. *B*, half-life of hPRTB is decreased in the presence of exogenous hRPF1/Nedd4. Results from three independent experiments were quantitated using a phosphorimager, normalized against an internal Myc-EGFP control, and expressed as percentage of labeled hPRTB protein at time 0. *C*, Western analysis of HeLa extracts expressing hRPF1/Nedd4, hRPF1/Nedd4-C867A, or an empty vector pcDNA3 control. Affinity-purified rabbit polyclonal antibody used for detection was raised against the hect domain of hRPF1/Nedd4.

the level of several cytoplasmic proteins (14, 15, 17). In this study, we have explored an additional role for Nedd4 with the identification and characterization of substrate proteins that are localized within the nucleus. In every aspect examined, the proline-rich nuclear protein, hPRTB, is a characteristic substrate for hRPF1/Nedd4 ubiquitination and degradation within the cell. However, given the lack of known determinants of *in vivo* WWhect specificity, we cannot exclude the possibility that there may be other human WWhect E3 ubiquitin ligases that are able to ubiquitinate this novel nuclear substrate within cells. Indeed, there are several human WWhect E3 ubiquitin ligases with similar domain structure to hRPF1/Nedd4, including Smurf1, a human WWhect ligase containing two WW domains, which targets Smad1 and Smad5 for ubiquitin-dependent degradation (9). However, our examination of Smurf1 amino acid sequence failed to identify either a bipartite nuclear localization sequence or rev-like export sequence similar to those in hRPF1/Nedd4, suggesting that certain WWhect enzymes, such as Smurf1, may specifically target substrates in the cytoplasm. Although our work strongly suggests a nuclear function for hRPF1/Nedd4, additional research into the *in vivo* substrate specificity of this and other potentially nuclear human WWhect proteins is necessary to address precise questions of overlapping enzyme/substrate choice.

The localization of a ubiquitination substrate such as hPRTB in nuclear speckles is not surprising, given observations that cellular proteins modified by the ubiquitin-like protein SUMO-1 are targeted to precise subnuclear localizations. SUMO-1-modified promyelocytic leukemia gene product (PML) localizes to nuclear bodies (38); similarly, the sumoylation of the homeodomain-interacting protein kinase 2 results in localization to nuclear speckles (dots) that are distinct from either splicing factor-rich speckles or PML bodies (39). The localization of hPRTB in nuclear speckles is unaffected by a mutation that blocks ubiquitination, suggesting that the covalent attachment of a ubiquitin moiety is not required for nuclear speckle localization. However, in addition to playing a significant role in subnuclear localization, SUMO-1 modification also antagonizes the ubiquitin-dependent degradation of proteins such as IκB, leading to an increase in protein stability (40). We acknowledge the possibility that hPRTB may also be targeted (by a distinct enzyme) for sumoylation, a ubiquitin-like modification, which could either direct its localization to nuclear speckles or antagonize its ubiquitin-dependent degradation. Further studies are needed to address whether hPRTB may be modified by such a ubiquitin-like protein.

Although the precise function of hPRTB remains unknown, its localization in nuclear speckles may offer clues to a possible

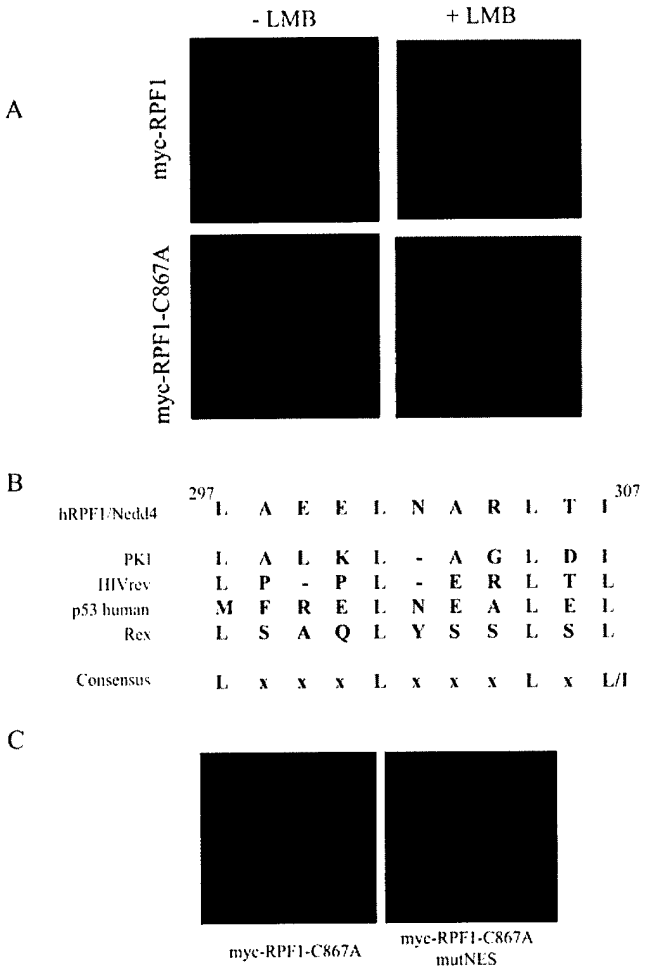

FIG. 6. **Amino acids 297–307 of hRPF1/Nedd4 contain a Rev-like nuclear export sequence.** *A,* HeLa cells were transfected with either Myc-hRPF1/Nedd4 or Myc-hRPF1/Nedd4-C867A, plated onto glass coverslips, and treated with leptomycin B (20 ng/ml) for 4 h. Localization of Myc-tagged constructs was detected using a Myc antibody (9E10), and Texas Red fluorescence was visualized using confocal microscopy. *B,* hRPF1/Nedd4 contains a consensus Rev-like NES. Amino acids 297–307 are aligned with the export sequences of PKI, HIVrev, human p53, and Rex. Conserved leucines within the consensus NES are highlighted in *blue*. *C,* mutation of the NES results in a steady state population of hRPF1/Nedd4-C867A in the nucleus. Two conserved residues within the NES of hRPF1/Nedd4 were substituted with alanine (L305A/I307A) within the context of the C867A catalytic mutant of Myc-tagged hRPF1/Nedd4. cDNAs were transiently transfected into HeLa cells, and cells were prepared for immunofluorescence as previously described.

function. Nedd4 was first described as a transcript that is dramatically down-regulated upon the maturation of neural precursor cells (11). Conversely, PRTB is a transcript that is highly expressed in the developing mouse inner ear and is present in high amounts in the adult brain (22). These reports of temporal and spatial expression of the Nedd4 and hPRTB transcripts are provocative given the results of this study. Given its colocalization with splicing factor-rich nuclear speckles, could hPRTB function as a developmental specific splicing factor? Initial attempts to demonstrate colocalization of core Sm proteins with hPRTB immunoprecipitates or alteration of a splice site choice *in vivo* by hPRTB (data not shown) were not successful. Although its cellular function remains unknown, it nonetheless remains possible that hPRTB may modulate a splicing or RNA processing event within cells.

Nuclear Import and Export of hRPF1/Nedd4—With our identification of a Rev-like NES within hRPF1/Nedd4, we provide evidence that Nedd4 can indeed access both cytoplasmic and nuclear compartments within a cell. However, it is apparent that hRPF1/Nedd4 protein that enters the nucleus has a very strong constitutive export sequence, resulting in little time spent resident within the nucleus. For example, exogenous hRPF1/Nedd4 targeted to the nucleus by a strong SV40 nuclear localization signal or a mutated nuclear export sequence is not tolerated by the cell, and protein does not accumulate (data not shown). Thus, although a population of hRPF1/Nedd4 is able to enter the nucleus, its presence appears to be transient, with the cell having a strong preference to return it to the cytoplasm. This transient nuclear localization of hRPF1/Nedd4 offers an additional explanation for our inability to isolate and immunoprecipitate what are presumably nuclear hRPF1/Nedd4-hPRTB complexes (data not shown).

Although hRPF1/Nedd4 is able to enter the nucleus, it is intriguing that neither leptomycin B treatment nor mutation of the NES is sufficient to "trap" all of the hRPF1/Nedd4 protein within the nucleus. Presumably the remaining cytoplasmic hRPF1/Nedd4 population has not received a signal for nuclear entry but instead may be poised to act upon known cytoplasmic Nedd4 targets, such as the sodium epithelial channel. Accordingly, one logical question raised is what stimulus or signal targets cytoplasmic hRPF1/Nedd4 to the nucleus? hRPF1/Nedd4 is cleaved in cells in response to apoptotic stimuli (41); however, a truncated Nedd4 protein corresponding to the caspase cleavage product retains a primarily cytoplasmic localization (data not shown), suggesting that removal of the first 200 amino acids is not a sufficient signal for nuclear import. Although other factors such as phosphorylation, acetylation, or a conformational change may signal hRPF1/Nedd4 nuclear import, regulation of Nedd4 localization may also occur at the level of nuclear export. For example, the sequences flanking the NES of hRPF1/Nedd4 could be modified or change conformation to specifically block accessibility of the NES to the export receptor, preventing efficient export. Such NES masking has been proposed to play a role in the blocking of p53 nuclear export upon p53 tetramerization (37). We have not yet observed a set of conditions under which hRPF1/Nedd4 is exclusively nuclear, but we remain interested in identifying factors that regulate such nuclear import and/or export. Experiments aimed at identifying developmental stages, cell types, or conditions under which hRPF1/Nedd4 may be localized within the nucleus will likely offer insight into the important role of nuclear/cytoplasmic localization in substrate recognition.

Given that hRPF1/Nedd4 may spend only a short time in the nucleus, it is likely that nuclear import of hRPF1/Nedd4 is the limiting step in enzyme/substrate recognition and catalysis. For example, an activating signal could effect the transient nuclear localization of hRPF1/Nedd4, resulting in ubiquitination of a substrate within the nucleus, followed by rapid export of hRPF1/Nedd4 to the cytoplasm. Alternatively, nuclear import of hRPF1/Nedd4 could be the limiting step if transient enzyme entry was needed to allow the enzyme to bind a nuclear substrate and transport it to the cytoplasm, where ubiquitination and degradation might occur. Such a piggyback mechanism is one explanation for the complex regulation of p53 by its RING-domain E3 ubiquitin ligase, MDM2, a protein demonstrated to shuttle to and from the nucleus (42, 43). Additional studies aimed at elucidating the location in the cell at which hRPF1/Nedd4 substrate binding and catalysis occur are needed to further our understanding of the localization constraints that affect *in vivo* enzyme/substrate regulation.

Cumulatively, this work establishes a novel role for Nedd4 within the nucleus, expanding our understanding of this E3 ubiquitin ligase as a regulator of both cytoplasmic and nuclear targets. Specifically, we have identified and characterized a

nuclear speckle-associated protein, hPRTB, as a substrate of hRPF1/Nedd4. Importantly, the identification of a nuclear export sequence within hRPF1/Nedd4 offers a mechanism by which a predominantly cytoplasmic enzyme accesses a nuclear substrate. Despite significant research efforts to date, there remains a paucity of knowledge of the *in vivo* determinants of substrate specificity for the WWhect E3 ubiquitin ligase family. With the identification of a nuclear import/export mechanism by which Nedd4 accesses a nuclear substrate, we propose that subcellular localization is an important component of *in vivo* WWhect enzyme/substrate recognition and regulation.

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Rsp5 Ubiquitin-Protein Ligase Mediates DNA Damage-Induced Degradation of the Large Subunit of RNA Polymerase II in *Saccharomyces cerevisiae*

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Rsp5 is an E3 ubiquitin-protein ligase of *Saccharomyces cerevisiae* that belongs to the hect domain family of E3 proteins. We have previously shown that Rsp5 binds and ubiquitinates the largest subunit of RNA polymerase II, Rpb1, in vitro. We show here that Rpb1 ubiquitination and degradation are induced in vivo by UV irradiation and by the UV-mimetic compound 4-nitroquinoline-1-oxide (4-NQO) and that a functional *RSP5* gene product is required for this effect. The 26S proteasome is also required; a mutation of *SEN3/RPN2* (*sen3-1*), which encodes an essential regulatory subunit of the 26S proteasome, partially blocks 4-NQO-induced degradation of Rpb1. These results suggest that Rsp5-mediated ubiquitination and degradation of Rpb1 are components of the response to DNA damage. A human WW domain-containing hect (WW-hect) E3 protein closely related to Rsp5, Rpf1/hNedd4, also binds and ubiquitinates both yeast and human Rpb1 in vitro, suggesting that Rpf1 and/or another WW-hect E3 protein mediates UV-induced degradation of the large subunit of polymerase II in human cells.

Ubiquitin-dependent proteolysis involves the covalent ligation of ubiquitin to substrate proteins, which are then recognized and degraded by the 26S proteasome. While many of the components involved in catalyzing protein ubiquitination have been identified and characterized biochemically, we are only beginning to understand how the system specifically recognizes appropriate substrates. At least three classes of activities, known as E1 (ubiquitin-activating), E2 (ubiquitin-conjugating), and E3 (ubiquitin-protein ligase) enzymes, cooperate in catalyzing protein ubiquitination (34). The enzymatic mechanisms and functions of the E1 and E2 proteins have been well characterized. In contrast, the E3 enzymes are a diverse and less-well-characterized group of activities, and many lines of evidence indicate that E3 activities play a major role in determining the substrate specificity of the ubiquitination pathway (14, 28, 34).

The hect (homologous to E6-AP carboxyl terminus) domain defines a family of E3 proteins that were discovered through the characterization of human E6-AP (17). The interaction of E6-AP with the E6 protein of the cervical cancer-associated human papillomavirus types causes E6-AP to associate with and ubiquitinate p53, suggesting that E6 functions in promoting cellular immortalization by, at least in part, stimulating the destruction of this important tumor suppressor protein (16). The hect E3 molecular masses range from 92 to over 500 kDa, with the hect domain comprising the approximately 350 carboxyl-terminal amino acids (17, 34). Exactly five hect E3s are encoded by the *Saccharomyces cerevisiae* genome, and over 30 have been identified so far in mammalian species. An obligatory intermediate in the ubiquitination reactions catalyzed by hect E3s is a ubiquitin-thioester formed between the thiol group of an absolutely conserved cysteine within the hect domain and the terminal carboxyl group of ubiquitin (33). E3 becomes "charged" with ubiquitin via a cascade of ubiquitin-thioester transfers, in which ubiquitin is transferred from the active-site cysteine of an E1 enzyme to the active-site cysteine of an E2 enzyme and finally to hect E3, which catalyzes isopeptide bond formation between ubiquitin and the substrate. E3 can apparently be recharged with ubiquitin while bound to the substrate and can therefore catalyze ligation of multiple ubiquitin moieties to the substrate, through conjugation either to other lysines on the substrate or to lysine residues on previously conjugated ubiquitin molecules. The resulting multiubiquitinated substrate is then recognized and degraded by the 26S proteasome. Structure-function analyses of human E6-AP and yeast Rsp5 have suggested a model for hect E3 function in which the large and nonconserved amino-terminal domains of these proteins contain determinants for substrate specificity, while the carboxyl-terminal hect domain catalyzes the multiubiquitination of bound substrates (16, 39).

The *S. cerevisiae RSP5* gene encodes an essential hect E3 protein, and mutations in the gene have been isolated in multiple genetic screenings, including one for a suppressor of mutations in *SPT3* (reference 41; also cited in references 17 and 18). Spt3 is part of the TATA-binding protein recognition component of the SAGA complex, which plays an important role in transcriptional activation in vivo and contains histone acetyltransferase activity (37). Rsp5 has also been identified as being involved in the down-regulation of several plasma membrane-associated permeases, including uracil permease (Fur4), general amino acid permease (Gap1), maltose permease (Mal61), and the plasma membrane H^+-ATPase (5, 9, 13, 23). The primary structure of yeast Rsp5 reveals, in addition to its carboxyl-terminal hect domain, two types of domains within the amino-terminal region: C2 (one domain between amino acids 3 and 140) and WW (three domains between amino acids 231 and 418). C2 domains interact with membrane phospho-

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lipids, inositol polyphosphates, and proteins, in most cases dependent on or regulated by Ca^{2+} (31). Although it has not yet been demonstrated, it is possible that the C2 domain of Rsp5 is involved in targeting its membrane-associated substrates either by localizing Rsp5 to the plasma membrane or by directly mediating the interactions with these substrates.

WW domains are protein-protein interaction modules that recognize proline-rich sequences, with the consensus binding site containing either a PPXY (4, 21), PPLP (1a, 7), or PPPGM (2) sequence. WW domains, like SH3 domains, recognize polyproline ligands with high specificity but low affinity (K_d = 1 to 200 μM). The basis of recognition is the N-substituted nature of the proline peptide backbone rather than the proline side chain itself (26). It has been suggested that this explains how WW and SH3 domains can achieve specific but low-affinity recognition of ligands, since proline is the only natural N-substituted amino acid. It has also recently been shown that WW domains can recognize phosphoserine- and phosphothreonine-containing ligands (22), which has important implications for the diversity of substrates that may be recognized by Rsp5 and other WW domain-containing hect E3s. A structure-function analysis of Rsp5 showed that the hect domain and the region spanning WW domains 2 and 3 are necessary and sufficient to support the essential in vivo function of Rsp5, while the C2 domain and WW domain 1 are dispensable, at least under standard growth conditions (39). Together, the results of our structure-function analyses imply that ubiquitination of one or more substrates of Rsp5 is essential for cell viability and that the critical substrate(s) is recognized by the region containing WW domains 2 and 3.

Members of our group previously reported the results of a biochemical approach for identifying substrates of Rsp5, which led to the identification of Rpb1, the largest subunit (LS) of RNA polymerase II (Pol II), as a substrate of Rsp5 (18). Rpb1 is very efficiently ubiquitinated by Rsp5 in vitro, and the WW domain region mediates binding to Rpb1, with WW domain 2 being most critical. Since the requirements for Rpb1 binding and ubiquitination parallel those for the essential function of Rsp5, Rpb1 is a candidate for being at least one of the substrates related to the essential function of Rsp5. The biological relevance of Rpb1 ubiquitination was not initially clear, however, since Rpb1 is an abundant, long-lived protein in vivo. Interestingly, another study showed that the Pol II LS is subject to ubiquitination and degradation in response to UV irradiation (3, 30); however, the enzymatic components of the ubiquitin system responsible for this phenomenon were not identified or characterized. We show here that UV irradiation or treatment with a UV-mimetic chemical induces the degradation of Rpb1 in yeast cells and that Rsp5 and the 26S proteasome mediate this effect. Furthermore, we show that human Rpf1, a WW domain-containing hect (WW-hect) E3 protein, binds and ubiquitinates Rpb1 in vitro, suggesting that this may be the E3 protein that mediates UV-induced degradation of the Pol II LS in human cells.

MATERIALS AND METHODS

Yeast strains and plasmids. FY56 (*RSP5*), FW1808 (*rsp5-1*), and the *Gal-RSP5* strain were described previously (18, 39). The *sen3-1* (MHY811) and *SEN3* (MHY810) strains (6) were kindly provided by Mark Hochstrasser (University of Chicago). The *tom1* null mutant strain was made by single-step gene disruption in the diploid strain W303, and haploid *tom1*Δ colonies were isolated by the sporulation and dissection of the heterozygous *TOM1/tom1*Δ diploid. All plasmids that promote the expression of Rsp5 and Rpb1 were described previously (18, 39). Plasmids that promote the bacterial expression of glutathione *S*-transferase (GST)–Rpf1 fusion proteins were generated by PCR amplification of regions of the Rpf1 open reading frame in plasmid pBKC-hRPF1 (19). The GST-Rpf1 N protein contains amino acids 13 to 192 of Rpf1, the GST-WW protein contains amino acids 193 to 506, the GST-C protein contains amino acids 506 to 901, and the GST–WW-hect protein contains amino acids 193 to 901. This numbering is based on the assumption that amino acid 29 of the protein sequence given in GenBank (accession no. D42055) is the initiating methionine. pGEX-5x-1 (Pharmacia, Piscataway, N.J.) was the cloning vector for the expression of all the GST fusion proteins except for GST–WW-hect, which was expressed by pGEX-6p-1.

Protein purification and biochemical assays. GST fusion proteins for ubiquitination assays and protein binding assays were expressed in *Escherichia coli* by standard methods and affinity purified on glutathione-Sepharose (Pharmacia). Ubiquitination assays utilized hect E3 proteins (Rsp5, the Rsp5 C-A mutant, human E6-AP, and Rpf1 WW-hect) that were cleaved from the GST portion of the molecule with PreScission protease (Pharmacia). These proteins were then used in ubiquitination assays with ^{35}S-labeled yeast Rpb1 that had been translated in vitro with a TNT rabbit reticulocyte lysate system (Promega, Madison, Wis.), as described previously (18).

Rpb1 binding assays were performed by mixing 100 ng of GST-E3 fusion protein bound to 10 μl of glutathione-Sepharose with 80 μg of total HeLa cell lysate (cell lysis buffer: 0.1 M Tris [pH 8.0], 0.1 M NaCl, and 1% NP-40), with the remainder of the 125-μl volume consisting of 25 mM Tris (pH 8.0) and 125 mM NaCl. Reaction mixtures were rotated for 2 h at 4°C, and the beads were washed three times with 500 μl of cell lysis buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer was added directly to the Sepharose and heated at 95°C for 5 min, and proteins were analyzed by SDS-PAGE and Western blotting with either anti-carboxyl-terminal domain (anti-CTD) antibody (generously provided by Danny Reinberg, University of Medicine and Dentistry of New Jersey, Piscataway) or anti-Pol II antibody N-20 from Santa Cruz Biotechnology (Santa Cruz, Calif.).

Analysis of UV- and 4-NQO-treated cells. HeLa cells were maintained in Dulbecco's modified Eagle medium with 10% fetal bovine serum, and UV irradiation was performed on tissue culture dishes after the removal of the medium. A germicidal lamp emitting light at 254 nm with an incident dose rate of 1.5 J per m^2 per s was used, and the time of irradiation was generally 15 s, for a total dose of 22.5 J per m^2. Fresh medium was then added to the cells, which were then allowed to recover for various times at 37°C. 4-Nitroquinoline-1-oxide (4-NQO) (Sigma), prepared as a 0.5-mg/ml stock solution in ethanol, was added directly to the medium at various concentrations and times. Extracts were made by lysing cells directly in SDS-PAGE loading buffer.

Yeast cells were irradiated as follows. Log-phase liquid cultures (5 optical density [OD] units) were concentrated by centrifugation to 0.5 ml, and then the cells were spread onto 10-cm agar plates. The liquid was allowed to absorb into the plates for 30 min at 30°C, and then the plates were irradiated for 15 s, as described above for HeLa cells. The cells were then collected from the plates and extracts were prepared as described below. Log-phase liquid yeast cultures (5 OD units) were treated with 4-NQO by adding a 0.5-mg/ml stock solution in ethanol directly to the culture medium for either 30 or 60 min. Yeast cell extracts were prepared by the method of Silver et al. (36). Briefly, 5 OD units of cells were resuspended in 1 ml of 0.25 M NaOH–1% β-mercaptoethanol and incubated on ice for 10 min. A volume of 0.16 ml of 50% trichloroacetic acid was added, and incubation on ice was continued for 10 min. The precipitate was collected by microcentrifugation at 4°C for 10 min, and then the pellet was washed with cold acetone, dried, and resuspended in 200 μl of SDS-PAGE sample buffer. Samples were heated at 95°C for 10 min prior to being loaded onto SDS-PAGE gels. Protein from the equivalent of 0.1 to 0.25 OD unit of cells was analyzed on SDS–7% PAGE gels for Western analyses of Rpb1. Immunoprecipitation and Western blotting (see Fig. 4) were performed by diluting 40 μl of yeast extract with 1.4 ml of 25 mM Tris (pH 7.9)–125 mM NaCl, followed by the addition of antibody and 20 μl of protein A-Sepharose (Pharmacia). The mixture was rotated at 4°C for 4 h; the Sepharose beads were collected, washed, and boiled in sample buffer; and then the proteins were analyzed by SDS-PAGE followed by immunoblotting.

Antibodies utilized in this study were either anti-CTD rabbit polyclonal antibody (used for yeast Rpb1 Western analyses and Rpb1 immunoprecipitations; generously provided by Danny Reinberg), anti-human Pol II rabbit polyclonal antibody N-20 (Santa Cruz Biotechnology), antiubiquitin mouse monoclonal antibody (Santa Cruz Biotechnology), antihemagglutinin rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-Rsp5 mouse monoclonal antibody (39), or anti-Rfa1 rabbit polyclonal antibody (generously provided by Steve Brill, Rutgers University). Horseradish peroxidase-linked secondary antibodies and chemiluminescent reagents were obtained from DuPont NEN.

RESULTS

UV irradiation and 4-NQO induce the degradation of Rpb1 in both human and yeast cells. 4-NQO is considered a UV mimetic because it is metabolized to yield a compound that reacts with purine nucleotides of DNA, and these adducts are processed by the nucleotide excision repair (NER) system in a manner similar to that of dipyrimidine photoproducts induced by 254-nm UV light (15, 29). It was previously shown that UV irradiation of human cells induces the ubiquitination and deg-

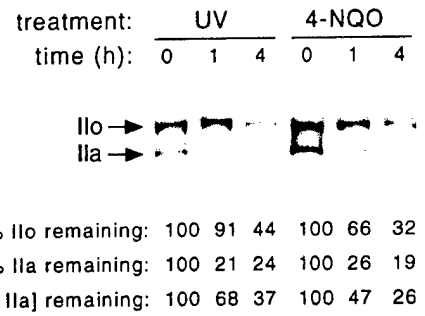

FIG. 1. hRpb1 levels following UV irradiation and 4-NQO treatment of HeLa cells. HeLa cells were irradiated with 254-nm UV light at 22.5 J per m^2 as described in Materials and Methods, and cell extracts were prepared immediately or 1 or 4 h postirradiation. For 4-NQO treatment, the chemical was added directly to the culture medium at a final concentration of 0.5 μg/ml, and cell extracts were prepared immediately or 1 or 4 h later. Relative hRpb1 levels were determined by SDS-PAGE and immunoblotting and quantitated by densitometry. Levels are expressed as the percentage of Rpb1 remaining relative to the level in untreated cells.

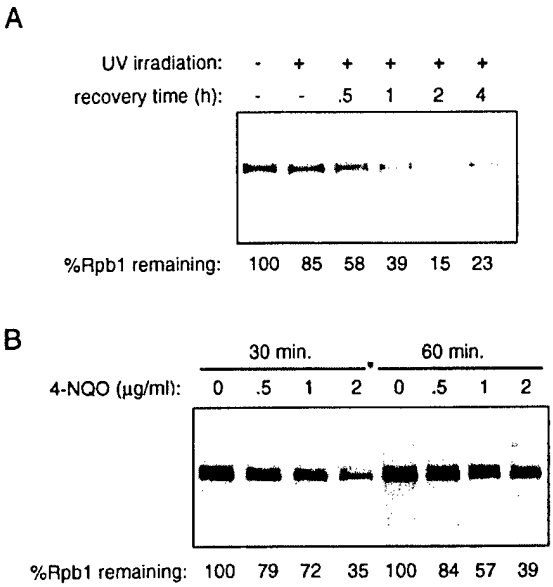

FIG. 2. (A) Rpb1 levels following UV irradiation of yeast. Yeast cells (strain FY56) were irradiated at 22.5 J per m^2 as described in Materials and Methods, and whole-cell extracts were made at the indicated times postirradiation. Rpb1 was detected by SDS-PAGE followed by immunoblotting with anti-CTD antibody. Rpb1 levels were quantitated by densitometry and are expressed as the percentage of Rpb1 remaining relative to the level in untreated cells. (B) Rpb1 levels following 4-NQO treatment. 4-NQO was added to liquid cultures of log-phase yeast at the indicated concentrations, and cells were collected at the indicated times following addition. Whole-cell extracts were prepared, and Rpb1 was detected by SDS-PAGE and immunoblotting.

radation of human Rpb1 (hRpb1) (3, 30). Figure 1 demonstrates this effect in HeLa cells. Cells were irradiated with 254-nm UV light at a dose of 22.5 J per m^2, and cell extracts were made at various times, up to 4 h after irradiation. Extracts were analyzed by SDS-PAGE, followed by immunoblotting with an antibody that recognizes the amino-terminal region of hRpb1 and therefore detects both hypophosphorylated (IIa) and hyperphosphorylated (IIo) forms of the protein. The degradation of the IIa form was more rapid and more complete than the degradation of the IIo form, with the IIa form reaching a minimum degradation of 20 to 25% of the initial amount after 1 h, while the IIa form reached a minimum degradation of 40 to 50% of the initial amount after 4 h. 4-NQO treatment stimulated the degradation of hRpb1 over a similar time course, again with the IIa form disappearing more rapidly and more completely than the IIo form. Lactacystin, a highly specific inhibitor of the proteasome, inhibited both UV- and 4-NQO-induced degradation of hRpb1 (not shown), which is consistent with previous reports that this effect is mediated by the 26S proteasome of the ubiquitin system (30).

Figure 2 shows that the degradation of Rpb1 was also induced in *S. cerevisiae* by both UV irradiation and 4-NQO treatment. UV irradiation of intact yeast cells on agar plates led to a dose- and time-dependent decrease in the steady-state level of Rpb1 (Fig. 2A). Rpb1 levels reached a minimum of 15 to 20% of the initial amount between 1 and 2 h after irradiation and began to return to normal after 4 h. 4-NQO also elicited a dose-dependent decrease in Rpb1 levels, reaching a minimum 30 to 60 min after the addition of 4-NQO (Fig. 2B). The amount of Rpb1 remaining in the experiment whose results are shown was 35 to 40% of the initial amount; however, in other experiments, the minimum was generally 25 to 30% (Fig. 3). Neither UV irradiation nor 4-NQO treatment resulted in a significant loss of viability at doses necessary to elicit maximal Rpb1 degradation. Unlike hRpb1, the hypo- and hyperphosphorylated forms of yeast Rpb1 migrate as a very closely spaced doublet and are not easily distinguished by SDS-PAGE. Therefore, it is difficult to conclude whether there is an apparent preferential disappearance of one form over the other, as there is with hRpb1.

To rule out the possibility that the decrease in yeast Rpb1 levels accompanying UV or 4-NQO treatment was simply the result of inhibition of the synthesis of Rpb1, 4-NQO-treated cells were compared to cells treated with cycloheximide. As shown in Fig. 3A, cycloheximide treatment led to only a slight decrease in Rpb1 levels after 45 min, whereas 4-NQO treatment resulted in the reduction in Rpb1 levels as described above. Total cellular protein levels were not affected by 4-NQO treatment, and Coomassie blue staining of SDS-PAGE gels indicated that the effect of 4-NQO was specific for Rpb1. This was confirmed by immunoblotting for an unrelated nuclear protein, Rfa1, a component of replication protein A. Figure 3B shows that levels of Rfa1 were not affected by 4-NQO treatment under conditions in which Rpb1 degradation was induced.

The appearance of slower-migrating forms of Rpb1, suggestive of ubiquitinated intermediates, was evident in some experiments at higher concentrations of 4-NQO and on longer film exposures. These slower-migrating bands were shown to be ubiquitinated forms of Rpb1 by immunoprecipitating them with anti-Rpb1 antibody, followed by immunoblotting with either anti-CTD or antiubiquitin antibody (Fig. 4). While the accumulation of ubiquitinated forms of Rpb1 was clearly stimulated by 4-NQO, there was some reaction of the Rpb1 immunoprecipitate with the antiubiquitin antibody even in untreated cells. This may reflect a basal level of Rpb1 ubiquitination in normally growing cells, as suggested previously (18).

4-NQO-induced degradation of Rpb1 is dependent on *RSP5* and *SEN3/RPN2*. Members of our group previously showed that Rsp5 ubiquitinates Rpb1 in vitro (18). To determine if the in vivo-induced degradation of Rpb1 was dependent on Rsp5, we first took advantage of a yeast strain that contains a single copy of a conditionally expressed wild-type *RSP5* gene. The

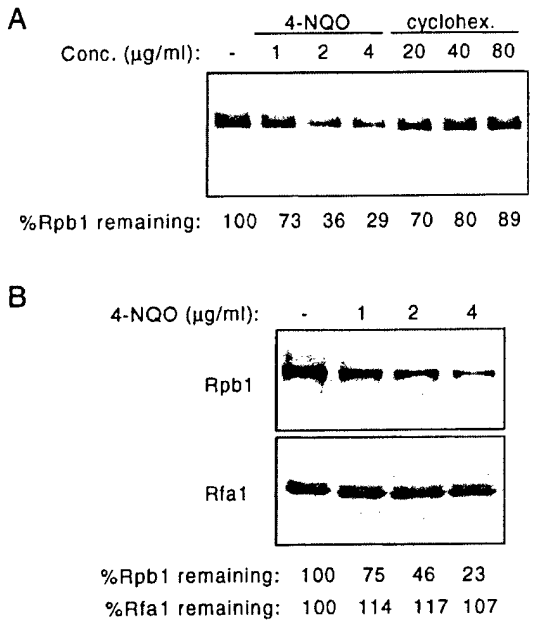

FIG. 3. (A) Yeast cells (FY56 [*RSP5*]) were treated with the indicated doses of 4-NQO or cycloheximide (cyclohex.) for 30 min, cell extracts were prepared, and Rpb1 levels were examined by SDS-PAGE and immunoblotting with anti-CTD antibody. (B) Yeast cells (FY56 [*RSP5*]) were treated with the indicated doses of 4-NQO for 30 min, cell extracts were prepared, and Rpb1 levels and Rfa1 levels were examined by SDS-PAGE and immunoblotting.

Gal-RSP5 yeast strain contains an epitope-tagged *RSP5* gene under the control of the *GAL1* promoter, which is integrated at the *RSP5* chromosomal locus (18). This strain was grown to early log phase in galactose-containing medium, and then it was switched to dextrose-containing medium for 48 h. Figure 5A shows that Rsp5 protein levels were dramatically reduced after 48 h in dextrose. The cells were still fully viable at this point and resumed growth when shifted back to galactose-containing medium. The dextrose-shifted cells were treated with 4-NQO and compared to log-phase cells that had been maintained in galactose-containing medium. 4-NQO-induced Rpb1 degradation occurred in the cells maintained in galactose, but not in Rsp5-depleted cells. These results suggest that 4-NQO-induced degradation of Rpb1 is dependent on *RSP5*.

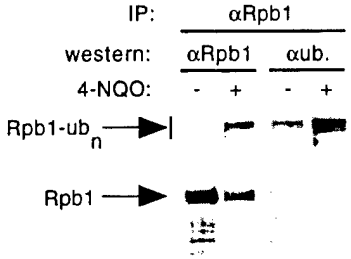

FIG. 4. Antiubiquitin antibody recognizes high-molecular-weight forms of Rpb1 from 4-NQO-treated cells. Yeast cells were treated with 4-NQO at 4 μg/ml for 30 min, and whole-cell extracts were prepared. Rpb1 was immunoprecipitated (IP) in duplicate from each sample with anti-CTD antibody. The immunoprecipitates were then analyzed by SDS-PAGE followed by immunoblotting with either anti-CTD (αRpb1) or antiubiquitin (αub.) antibody.

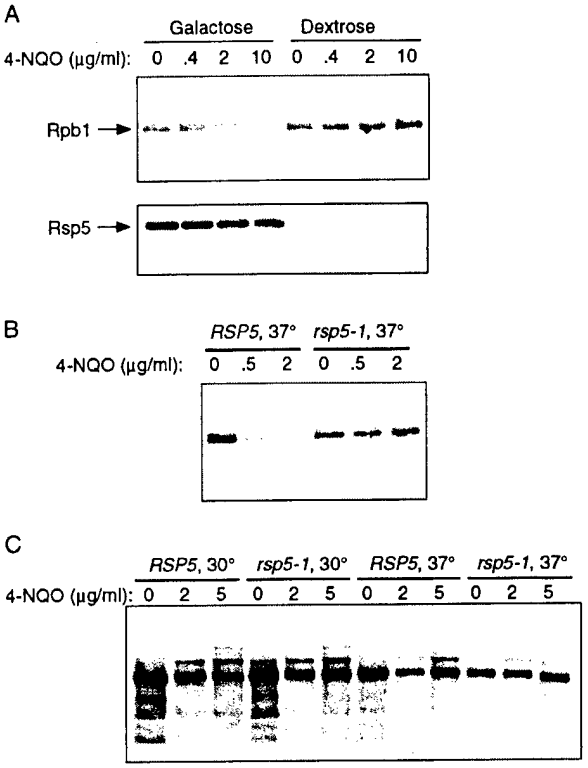

FIG. 5. (A) 4-NQO treatment of the *Gal-RSP5* strain maintained in galactose or shifted to dextrose. The *Gal-RSP5* strain was grown to early log phase in galactose-containing medium, and then the cells were either shifted to dextrose-containing medium for 48 h or maintained in galactose-containing medium. The cultures were then treated with 4-NQO at the indicated concentrations for 30 min, and whole-cell extracts were prepared and analyzed by SDS-PAGE and immunoblotting with either an anti-Rsp5 monoclonal antibody (bottom) or anti-CTD antibody (top). (B) 4-NQO treatment of the *rsp5-1* temperature-sensitive mutant. Strains FY56 (*RSP5*) and FW1808 (*rsp5-1*) were grown to mid-log phase at 30°C and then shifted to 37°C for 1 h. 4-NQO was then added at the indicated concentrations for 30 min. Whole-cell extracts were prepared, and Rpb1 was detected by SDS-PAGE and immunoblotting. (C) Experiment similar to that in panel B, except that cells were treated with 4-NQO at both 30 and 37°C.

To independently confirm the importance of Rsp5 in the induced degradation of Rpb1, we examined the effect of 4-NQO on the temperature-sensitive *rsp5-1* mutant. Temperature sensitivity is conferred by a single amino acid change (amino acid 733) within the hect domain that directly affects the catalytic activity of the protein (39). The *rsp5-1* strain grows with a slightly longer doubling time than an isogenic *RSP5* strain at 30°C but arrests within 30 to 60 min after a shift to 37°C. Figure 5B shows that Rpb1 degradation was induced by 4-NQO in an isogenic wild-type *RSP5* strain at 37°C, while little or no loss of Rpb1 was seen in the *rsp5-1* strain at 37°C. Figure 5C shows the results of an experiment in which multi-ubiquitinated forms of Rpb1 were evident following 4-NQO treatment. The accumulation of these forms was seen in the wild-type *RSP5* strain at both 30 and 37°C and in the *rsp5-1* strain at 30°C, but not at 37°C. These results again indicate that 4-NQO-induced ubiquitination and degradation of Rpb1 are *RSP5* dependent.

A strain containing a mutation in a subunit of the 26S pro-

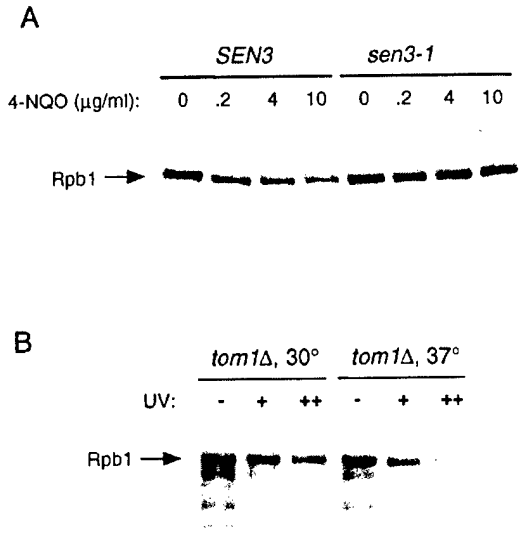

FIG. 6. (A) 4-NQO treatment of *SEN3* and *sen3-1* strains at 37°C. 4-NQO was added at the indicated concentrations for 30 min. Whole-cell extracts were prepared, and Rpb1 was detected by SDS-PAGE and immunoblotting. (B) The *tom1Δ* mutant was grown at 30°C and then either maintained at 30°C or shifted for 4 h to 37°C. Cells were then irradiated at either 25 (+) or 50 (++) J/m^2, followed by a 1-h recovery period at their respective temperatures. Whole-cell extracts were then prepared, and Rpb1 was detected by SDS-PAGE and immunoblotting.

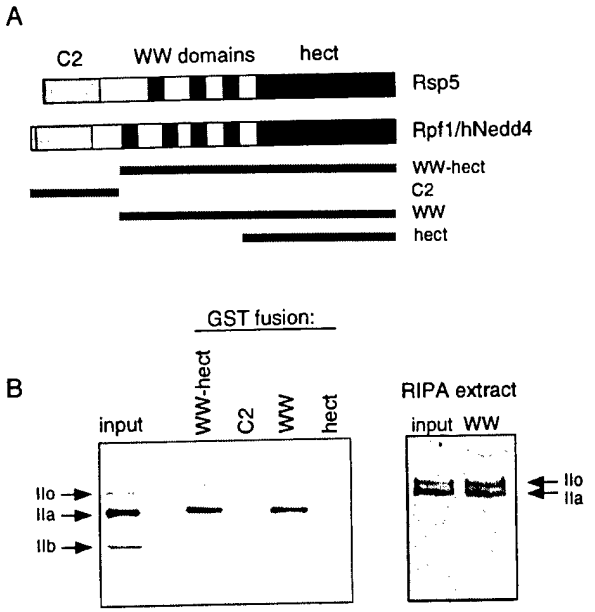

FIG. 7. (A) Schematic representation of yeast Rsp5 and human Rpf1/Nedd4. GST-Rpf1 fusions to the regions of Rpf1 indicated by the solid bars were made. (B) (Left) HeLa cell extract was prepared in NP-40 lysis buffer (see Materials and Methods). The binding of hRpb1 to GST-Rpf1 fusion proteins immobilized on glutathione-Sepharose was analyzed by SDS-PAGE and immunoblotting. The "input" shows hRpb1 in the extract with forms IIo, IIa, and IIb. (Right) Similar experiment, with HeLa cell extract prepared in radioimmunoprecipitation assay (RIPA) buffer. The input and binding to GST-WW are shown.

teasome was used to determine if the 4-NQO-induced degradation of Rpb1 was proteasome dependent. *SEN3/RPN2* encodes an essential non-ATPase regulatory subunit of the 26S proteasome (6). The *sen3-1* mutant shows a growth defect at 30°C (doubling time of 4.5 h) and a more severe growth defect at higher temperatures. The MATα2 transcription factor and certain artificial substrates of the ubiquitin system (Ub-Pro-β-galactosidase and Ub-Leu-β-galactosidase) have been shown to be stabilized in this mutant at 30°C. We compared the *sen3-1* mutant to an isogenic wild-type *SEN3* strain for its ability to support 4-NQO-induced degradation of Rpb1. As shown in Fig. 6A, the *sen3-1* mutant was defective in 4-NQO-induced degradation of Rpb1 compared to the *SEN3* strain. This result indicates that UV-induced degradation of Rpb1 is proteasome dependent, consistent with the observation that proteasome inhibitors blocked the degradation of the human Pol II LS in response to UV irradiation (30).

A caveat to the experiments utilizing the *GAL-RSP5, rsp5-1*, and *sen3-1* strains is that both *RSP5* and *SEN3/RPN2* are essential genes, and their inactivation results in growth inhibition. Therefore, indirect effects cannot be ruled out as being responsible for the block in 4-NQO-induced Rpb1 degradation seen in these mutants. To rule out the possibility that the block in Rpb1 degradation is due to a general growth arrest, we examined a temperature-sensitive mutation in a gene not predicted to affect either Rsp5 or Rpb1. We examined a *tom1* null mutant, since *TOM1* encodes a hect E3 protein that does not interact with Rpb1. Interestingly, Tom1 appears to influence transcription through effects on ADA coactivators, possibly by targeting the Spt7 protein for ubiquitination (32). The *tom1* null mutant has a near-normal doubling time at 30°C but exhibits a strong growth arrest within 2 h after a shift to 37°C. The *tom1* mutant was UV irradiated either at 30°C or 4 h after a shift to 37°C, and Rpb1 levels were examined. Figure 6B shows that the degradation of Rpb1 was induced at both temperatures. Therefore, the lack of induced degradation in the *rsp5* and *sen3* mutants is unlikely to be due to general growth arrest or cell stress.

Rpf1/hNedd4, a human hect E3 protein related to Rsp5, binds and ubiquitinates Rpb1 in vitro. Rpf1, also known as human Nedd4 (hNedd4), has a C2 domain at its extreme amino terminus, four WW domains in the central portion of the molecule, and a carboxyl-terminal hect domain (Fig. 7A). Rpf1 is one of at least seven human hect E3s that have this general organization, with a variable number of WW domains (two to four). GST-Rpf1 proteins were expressed as indicated in Fig. 7A, and equivalents amounts (100 ng) of each protein were assayed for the ability to bind to hRpb1. The full-length Rpf1 protein was not used in this analysis because it was produced in small amounts in bacteria and, furthermore, was not catalytically active, as judged by ubiquitin-thioester assays (not shown). Rpf1 WW-hect and the isolated WW domain region stably bound the hRpb1 present in the HeLa cell extract (Fig. 7B, left panel), whereas neither the isolated C2 domain nor the hect domain bound to hRpb1. These results are consistent with previous results showing that the WW domain region of Rsp5 is necessary and sufficient for binding to yeast Rpb1 (18, 39). In addition, a well-characterized proteolyzed form of hRpb1 (form IIb) that lacks the CTD did not bind to Rpf1, also consistent with previous results showing that the CTD is the binding site for Rsp5 (18, 39). There was an apparent preferential binding of Rpf1 to the hypophosphorylated (IIa) form of hRpb1 in this experiment; however, the degree to which the phosphorylated (IIo) form of hRpb1 associated with Rpf1 was dependent on the cell extraction buffer. When the cell lysis buffer conditions were harsher (radioimmunoprecipitation assay buffer instead of NP-40 lysis buffer [11]), an equiv-

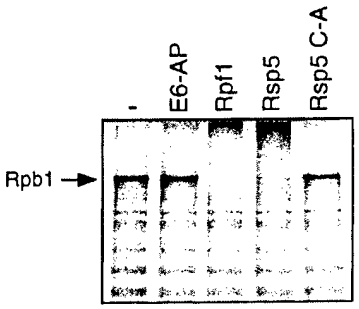

FIG. 8. Ubiquitination of Rpb1 by Rpf1 in vitro. Rpb1 was translated in vitro in rabbit reticulocyte lysate in the presence of [^{35}S]-methionine. Purified hect E3 proteins (human E6-AP, human Rpf1 [WW-hect; amino acids 193 to 901], yeast Rsp5, and the mutant of Rsp5 with a change of the active-site Cys to Ala [C-A]) were incubated as indicated with Rpb1 in the presence of ATP, ubiquitin, E1 enzyme, and E2 enzyme (*Arabidopsis thaliana* Ubc8) as previously described (18).

alent portion of hyperphosphorylated hRpb1 bound to Rpf1 (Fig. 7B, right panel). This suggests that the interaction of the hyperphosphorylated CTD with other proteins might preclude binding to Rpf1 and that Rsp5 and Rpf1 have an inherent ability to bind to both forms of the protein. This interpretation is consistent with previous results showing that Rsp5 could bind to both the IIo and IIa forms of purified Pol II holoenzyme in vitro (18).

To determine if Rpf1 can ubiquitinate Rpb1, the Rpf1 WW-hect protein was cleaved from the purified GST fusion protein and assayed for its ability to ubiquitinate in vitro-translated yeast Rpb1. Rpf1 was as efficient in stimulating multiubiquitination of Rpb1 as yeast Rsp5 (Fig. 8). Neither the mutant of Rsp5 with a change of the active-site cysteine to alanine nor human E6-AP ubiquitinated Rpb1. Together, the binding and ubiquitination results suggest that Rpf1 may mediate the DNA damage-induced degradation of the Pol II LS in human cells.

DISCUSSION

Rpb1 was initially identified as a substrate of Rsp5 based on a biochemical screening for proteins that were bound and ubiquitinated by Rsp5 in vitro (18). While Rsp5 was found to efficiently multiubiquitinate Rpb1 in vitro, the biological function of this was unclear, since Rpb1 is an abundant and stable protein in vivo. The steady-state level of Rpb1 was found to increase modestly (approximately three- to fivefold) on prolonged transcriptional repression of *RSP5*, providing evidence that Rpb1 may be a bona fide substrate of Rsp5 in vivo, even if the half-life of Rpb1 under normal growth conditions is relatively long. Other studies have shown that the inhibition of transcription caused by the exposure of mammalian cells to DNA-damaging agents or treatment, including α-amanitin, actinomycin D, cisplatin, and UV irradiation, leads to the degradation of the Pol II LS (3, 27). Ratner et al. further demonstrated that the degradation of the Pol II LS induced by UV irradiation was ubiquitin and proteasome dependent (30). Together, these results suggested that the recognition of Rpb1 by Rsp5 might be enhanced in response to DNA damage. The experiments described here showed that, as in human cells, DNA damage induces the ubiquitination and degradation of Rpb1 in *S. cerevisiae* and that this is dependent on the Rsp5 ubiquitin-protein ligase. In addition, a human hect E3 protein closely related to Rsp5, Rpf1/hNedd4, is shown to bind and ubiquitinate Rpb1 in vitro, suggesting that this hect E3 protein might mediate UV-induced degradation of Rpb1 in human cells.

It has long been recognized that RNA synthesis is down-regulated in response to DNA damage and that stalled RNA polymerase at sites of DNA damage might serve as a signal for the recruitment of the NER machinery (10, 24). This is thought to be the basis of a specialized form of NER, transcription-coupled repair (TCR), in which lesions within the transcribed strand of genes are repaired more rapidly than lesions on the nontranscribed strand or outside of the transcription units. TCR also occurs in *E. coli*, where the transcription repair coupling factor binds to and releases RNA polymerase stalled at a lesion and then stimulates the recruitment of the repair machinery (35). Several lines of evidence suggest that the mechanism of TCR is more complex in eukaryotes, and it is generally thought that a stalled RNA polymerase can resume transcript synthesis following repair. This is based in part on the stability of stalled RNA polymerase-template-RNA complexes in vitro and the idea that it would be energetically wasteful to abort transcript synthesis entirely. The finding that a fraction of the Pol II LS is ubiquitinated and degraded in response to DNA damage suggests an alternative mechanism for the down-regulation of transcription in response to DNA damage: irreversible disassembly of transcription complexes by the degradation of the major catalytic subunit of Pol II.

It is not yet clear which form of Pol II is targeted for ubiquitin-mediated degradation following DNA damage. The CTD, which is necessary and sufficient for Rsp5 binding, is subject to phosphorylation and dephosphorylation events during the transcription cycle and is also the site of interaction of many components of the transcription machinery (25). The CTD is hypophosphorylated (IIa) in Pol II transcription initiation complexes and undergoes phosphorylation upon promoter clearance to yield a hyperphosphorylated (IIo) form that persists throughout transcription elongation. Ratner et al. (30) reported that ubiquitinated forms of hRpb1 detected after UV irradiation reacted with an antibody that is specific for the hyperphosphorylated form of hRpb1, suggesting that Pol II complexes arrested at intragenic damage sites might be the preferential substrate for ubiquitination. This is not consistent, however, with the observation that the hypophosphorylated form of hRpb1 preferentially disappears in response to either UV irradiation or 4-NQO treatment. In order to explain this discrepancy, Ratner et al. suggested that the apparent loss of hypophosphorylated hRpb1 upon UV irradiation might reflect a rapid conversion of hypo- to hyperphosphorylated Rpb1 in order to compensate for the loss of hyperphosphorylated Rpb1. While we cannot exclude this possibility, the data are also consistent with a model in which the hypophosphorylated form of Pol II is actually the preferential substrate for ubiquitination but that the kinetics of its ubiquitination and degradation are too rapid to allow the detection of ubiquitinated intermediates.

While further studies are clearly necessary to determine which form of Pol II is targeted for ubiquitin-mediated degradation in response to DNA damage in vivo, our in vitro results suggest that there is not a specific requirement for the recognition of Rpb1 by Rsp5 in terms of the phosphorylation state of the CTD. Phosphorylation of the CTD is not a prerequisite for Rsp5 recognition, since in vitro-translated Rpb1 and GST-CTD produced in bacteria are both efficiently recognized by Rsp5. We also showed previously that the hypo- and hyperphosphorylated forms of purified human Pol II holoenzyme bind equally well to GST-Rsp5 (18). In addition, both Rsp5 and Rpf1 bind to the hypophosphorylated form of hRpb1 present in human cell extracts; however, the degree to which

Rsp5 and Rpf1 can bind to hyperphosphorylated hRpb1 is a function of the cell extraction buffer, with more stringent extraction buffers resulting in more binding of the hyperphosphorylated forms. Together, these results suggest that the association of other transcription factors with Pol II, and specifically with the CTD, might block recognition by Rsp5 in vivo. Changes in Pol II transcription complexes in response to DNA damage, such as the dissociation of specific CTD-associated proteins or the dissociation of the elongated polymerase complex from the template, might then allow Rsp5 to bind and ubiquitinate Rpb1.

Rsp5 is the only hect E3 protein in yeast that has a C2 domain and WW domains, while at least seven human hect E3s with C2 and WW domains have been identified. The WW domains, as well characterized protein-protein interaction modules, are likely to mediate the interaction with at least some of the substrates of Rsp5, including Rpb1 (39). WW domains bind proline-rich ligands, with the best-characterized ligand being the PY motif (containing a PPXY sequence). In addition, it has recently been shown that WW domains can also recognize phosphoserine- and phosphothreonine-containing ligands (22), suggesting that there are two disparate types of WW domain ligands. The CTD heptapeptide consensus (YSPTSPS) may be a nonconsensus PY motif in the context of the repeating heptapeptide (YXPXXPXYXPXXPX). Alternatively, if the phosphorylated form of Rpb1 is the in vivo substrate of Rsp5, phosphorylation at the serine and/or threonine residues may contribute to recognition, although as mentioned above, phosphorylation is not required for the binding of Rsp5 to the CTD in vitro. Our finding that Rpf1/hNedd4 can bind and ubiquitinate hRpb1 in vitro suggests that this may be the E3 enzyme responsible for this effect in human cells. Preliminary results, however, indicate that other WW-hect E3s can also bind to Rpb1 in vitro (1). It is possible that while several of the WW-hect E3s can bind and ubiquitinate Rpb1 in vitro, intracellular localization is the key determinant of which E3 can target Rpb1 in vivo. Mouse Nedd4 and yeast Rsp5 are primarily cytoplasmic (12, 40); however, there is now a precedent for the ubiquitin-mediated degradation of nuclear proteins being linked to their export from the nucleus to the cytoplasm (8, 38).

While it is now established that DNA damage induces the degradation of Rpb1 in both yeast and human cells, the relevance of this to DNA repair is not yet clear. Rsp5 mutants do not show any apparent UV sensitivity, although we cannot yet rule out more subtle effects of Rsp5 on the efficiency of DNA repair. The fact that both CSA and CSB Cockayne syndrome cells were found to be defective in UV-induced Rpb1 degradation in human cells suggested that this is related to the process of TCR. However, a *rad26* null mutant (Rad26 is the yeast CSB homolog and the only yeast protein known to be required for TCR but not for NER) exhibited no defect in 4-NQO-induced Rpb1 degradation (data not shown). This suggests that TCR may not be directly linked to DNA damage-induced degradation of Rpb1, at least in yeast, and again raises the question of which form of Pol II is the in vivo substrate of Rsp5. The expression of Rpf1/hNedd4 in yeast cannot functionally substitute for expression by *RSP5* in terms of either cell viability or the UV-induced effect on Rpb1 (data not shown). The basis of this noncomplementation is not known but could be related to an inability of Rpf1 to productively interact with other components of the ubiquitin system in yeast.

Several examples of regulated substrate ubiquitination have now been characterized. In many cases, modification of the substrate, often by phosphorylation, can serve as a signal for recognition by specific E3 ubiquitin-protein ligases, as in the recognition of phosphorylated Sic1 by SCF^{Cdc4} (28). In other cases, the unmasking of ubiquitination signals can occur when a substrate dissociates from an interacting protein, as in the case of the mutual destruction of the MATα2 and MATa1 transcription factors upon the dissociation of the heterodimer (20). An unmasking of the recognition signals on Rpb1 in response to DNA damage may account for the observations that Rpb1 is freely and efficiently recognized by Rsp5 under several different experimental conditions in vitro yet is normally a stable and long-lived protein in vivo. It seems likely that the nature of the Rpb1 CTD, as an organizational center for many components of the basal transcription machinery, might preclude Rsp5 from interacting with Rpb1 during the normal transcription cycle. DNA damage may signal alterations in Pol II complexes in a manner that allows Rsp5 to recognize and ubiquitinate Rpb1. Further studies on the effects of DNA damage of Pol II holoenzyme complexes will aid in addressing this hypothesis.

ACKNOWLEDGMENTS

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Peptide Antagonists of the Human Estrogen Receptor

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Estrogen receptor α transcriptional activity is regulated by distinct conformational states that are the result of ligand binding. Phage display was used to identify peptides that interact specifically with either estradiol- or tamoxifen-activated estrogen receptor α. When these peptides were coexpressed with estrogen receptor α in cells, they functioned as ligand-specific antagonists, indicating that estradiol-agonist and tamoxifen–partial agonist activities do not occur by the same mechanism. The ability to regulate estrogen receptor α transcriptional activity by targeting sites outside of the ligand-binding pocket has implications for the development of estrogen receptor α antagonists for the treatment of tamoxifen-refractory breast cancers.

About 50% of all breast cancers express the estrogen receptor α (ERα) protein and recognize estrogen as a mitogen (*1*). In a subpopulation of these tumors, antiestrogens, compounds that bind ER and block estrogen action, effectively inhibit cell growth. In this regard, the antiestrogen tamoxifen has been widely used to treat ER-positive breast cancers (*2*). Although antiestrogen therapy is initially successful, most tumors become refractory to the antiproliferative effects of tamoxifen within 2 to 5 years. The mechanism by which resistance occurs is controversial; however, it does not appear to result as a consequence of ER mutations or altered drug metabolism (*3*). It may relate instead to the observation that tamoxifen is a selective estrogen receptor modulator (SERM), functioning as an ER agonist in some cells and as an antagonist in others (*4*). Consequently, the ability of tumors to switch from recognizing tamoxifen as an antagonist to recognizing it as an agonist has emerged as the most likely cause of resistance. Upon binding ER, both estradiol and tamoxifen induce distinct conformational changes within the ligand-binding domain (*5*). The tamoxifen-induced conformational change may expose surfaces on the receptor that allow it to engage the general transcription machinery. We used phage display to identify specific peptides that interacted with the estradiol- and tamoxifen-ER complexes and used these peptides to show that estradiol and tamoxifen manifest agonist activity by different mechanisms.

Affinity selection of phage-displayed peptide libraries was performed to identify peptides that could interact specifically with the agonist [17β-estradiol (estradiol) or 4-OH tamoxifen (tamoxifen)], activated ERα, or ERβ (*6*). Representative peptides from each of four classes presented in this study are shown in Fig. 1A. Several peptides that were isolated with estradiol-activated ERα (represented by α/β I) contained the Leu-X-X-Leu-Leu motif found in nuclear receptor coactivators (*7*). α II was isolated with either estradiol- or tamoxifen-activated ERα. Two classes of peptides, α/β III and α/β V, that interact specifically with tamoxifen-activated ERα and ERβ, respectively, were identified. The α/β V peptide was subsequently shown to interact with tamoxifen-activated ERα (*6*). Several additional peptides homologous to α/β V were identified. A BLAST search of the National Center for Biotechnology Information database with the derived consensus of the α/β V peptide class revealed that the yeast protein RSP5 and its human homolog, receptor potentiating factor (RPF1), both contain sequences homologous to α/β V. These proteins were previously shown to be coactivators of progesterone receptor B (PRB) transcriptional activity (*8*).

Peptide-peptide competition studies were performed with time-resolved fluorescence (TRF) to determine if the α II, α/β III, and α/β V peptides were binding the same or distinct "pockets" on the tamoxifen-ERα complex (*9*). The α/β III and α/β V peptides cross compete, and at equimolar peptide concentrations, 50% inhibition is observed (Fig. 1B). This result indicates that these two peptides bind to the same or overlapping sites on ERα. We believe that the α II peptide binds to a unique site as its binding was not competed by α/β V and only 50% inhibited by a 10-fold excess of the α/β III peptide.

We next assessed whether the peptides interacted with ERα in vivo using the mammalian two-hybrid system (*10*). The α/β I peptide interacted with ERα in the presence of the agonist estradiol but not the SERMs tamoxifen, raloxifene, GW7604, idoxifene, and nafoxidine or the pure antagonist ICI 182,780 (Fig. 2). The failure of antiestrogen-activated ERα to interact with the α/β I peptide is consistent with previ-

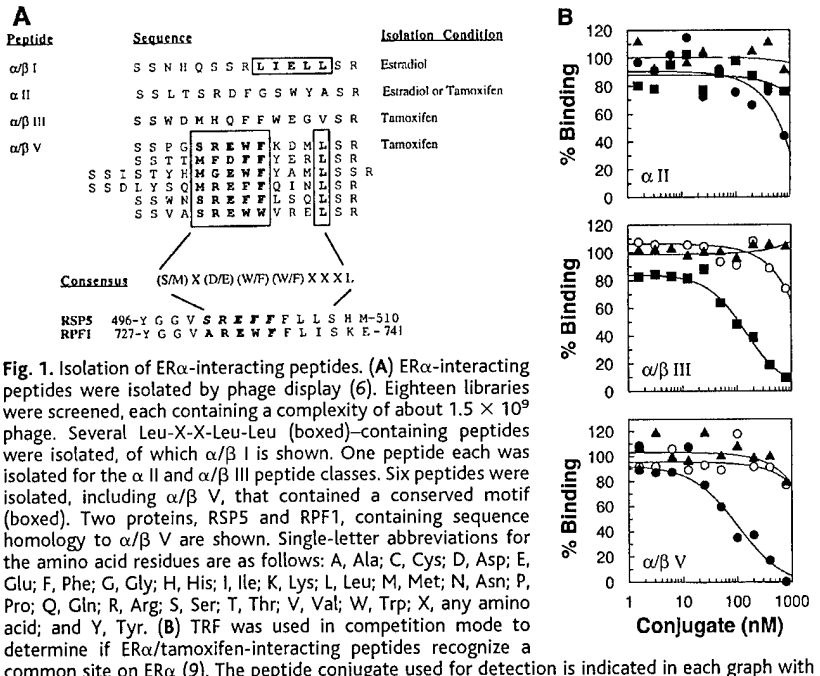

Fig. 1. Isolation of ERα-interacting peptides. (**A**) ERα-interacting peptides were isolated by phage display (*6*). Eighteen libraries were screened, each containing a complexity of about 1.5×10^9 phage. Several Leu-X-X-Leu-Leu (boxed)–containing peptides were isolated, of which α/β I is shown. One peptide each was isolated for the α II and α/β III peptide classes. Six peptides were isolated, including α/β V, that contained a conserved motif (boxed). Two proteins, RSP5 and RPF1, containing sequence homology to α/β V are shown. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; X, any amino acid; and Y, Tyr. (**B**) TRF was used in competition mode to determine if ERα/tamoxifen-interacting peptides recognize a common site on ERα (*9*). The peptide conjugate used for detection is indicated in each graph with the competing peptides as follows: ▲, no competitor; ○, α II; ●, α/β III; and ■, α/β V.

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ous studies that predict that the molecular mechanism of antagonism results from a structural change in the receptor ligand-binding domain that prevents coactivators from binding (*5*). α II interacted with the receptor in the presence of all modulators tested, with the unliganded (vehicle) and ICI 182,780–bound receptors showing the least binding activity. α/β III and α/β V interacted almost exclusively with the tamoxifen-bound ERα. ERα did not interact with the Gal4 DNA-binding domain (DBD) (control) alone in the presence of any modulators tested. Further studies indicated that binding of α II, α/β III, and α/β V occurs within the hormone-binding domain between amino acids 282 and 535 (*11*) and, unlike binding of α/β I, does not require a functional activation function 2 (AF-2) (www.sciencemag.org/feature/data/1039590.shl). These data indicate that SERMs induce different conformational changes in ERα within the cell and firmly establish a relation between the structure of an ERα-ligand complex and function.

When we examined the specificity of interaction between the peptides and heterologous nuclear receptors, we found, as expected, that the α/β I peptide interacted with ERβ, PRB, and the glucocorticoid receptor (GR) when bound by the agonists estradiol, progesterone, and dexamethasone, respectively (Fig. 3, A, B, and C). The α/β V peptide interacted with tamoxifen-bound ERβ and unexpectedly with PRB in the presence of the antagonists RU 486 or ZK 98299 (Fig. 3, A and B). The α/β V peptide, however, did not interact with the GR when bound by RU 486 or ZK 98299. α II and α/β III peptides failed to interact with ERβ, PRB, or GR.

We next tested the ability of the peptide-Gal4 fusion proteins to inhibit ERα transcriptional activity. Tamoxifen displayed partial agonist activity when analyzed with the ER-responsive complement 3 (C3) promoter in HepG2 cells (Fig. 4A). This activity can reach 35% of that exhibited by estrogen and is mediated by three nonconsensus estrogen response elements (EREs) located in the C3 promoter (*12*). When expressed in this system, the α/β I and α II peptides inhibited the ability of estradiol to activate transcription up to 50% and 30%, respectively (Fig. 4B). Two copies of the Leu-X-X-Leu-Leu sequence found in α/β I enhanced the inhibitory effect of this peptide and blocked estradiol-mediated transcription by about 90% (*13*). The inability of α/β III and α/β V to block estradiol-mediated transcription correlates well with their inability to bind the receptor when bound by agonist. Expression of α II, α/β III, and α/β V peptides blocked the partial agonist activity of tamoxifen (Fig. 4C). α II and α/β V were the most efficient disruptors of tamoxifen-mediated transcription, inhibiting this activity by about 90%. All peptide-Gal4 fusion proteins were expressed at similar levels, indicating that the relative differences in inhibition are not due to peptide stability (*11*). We also demonstrated that receptor stability and DNA binding are not affected by peptide expression (*11*). As expected, α/β I was unable to inhibit tamoxifen-mediated transcription. These findings are in agreement with the binding characteristics of these peptides and suggest that the pocket or pockets recognized by α II, α/β III, and α/β V are required for tamoxifen partial agonist activity. Although α/β V was shown to interact with PRB when bound by RU 486 (Fig. 3B), it was unable to block the partial agonist activity mediated by PRB/RU 486 (*11*). This result suggests that ERα/tamoxifen and PRB/RU 486 partial agonist activities are manifested differently. However, because α/β V was selected against ERα, this peptide may not bind PRB with high enough affinity to permit it to be useful as a PRB peptide antagonist.

Finally, we examined the ability of these peptides to inhibit ER transcriptional activity mediated through AP-1–responsive genes. This pathway has been proposed to account for some of the cell-specific agonist activity of tamoxifen (*14*). Both estradiol and tamoxifen activated transcription from the AP-1–responsive collagenase reporter gene, pCOL-Luc (Fig. 4D). This activity is manifest in the absence of an ERE and is believed to occur through a mechanism involving an interaction between ERα and the promoter-bound AP-1 complex (*14*). Regardless of the mechanism, each peptide was able to inhibit ERα-mediated transcriptional activity in a manner that reflected its ability to interact with the receptor in a ligand-dependent manner (Fig. 4E).

The mechanism by which tamoxifen manifests SERM activity is not yet known. Evidence presented in this study suggests that the tamoxifen-bound receptor exposes a binding site that is occupied by a coactivating protein not primarily used by the estradiol-activated receptor. The α II peptide, which interacts with both estradiol- and tamoxifen-bound receptors, inhibits the partial agonist activity of tamoxifen efficiently, while minimally affecting estradiol-mediated transcription. This result suggests that this site, although crucial for tamoxifen-mediated transcription, is dispensable for estrogen action. In addition, the ability of α/β III and α/β V to bind tamoxifen-specific surfaces and inhibit tamoxifen-mediated partial agonist activity suggests that these peptides may potentially recognize a protein contact site on ER that is critical for this activity. In this regard, we can

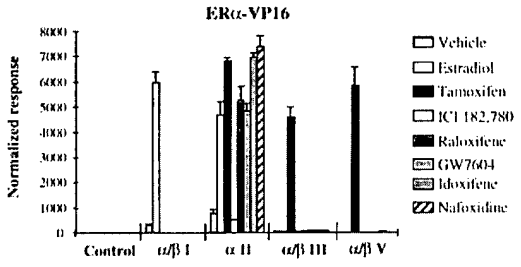

Fig. 2. ERα-peptide interactions in mammalian cells. The coding sequence of a peptide representative from each class identified was fused to the DBD of the yeast transcription factor Gal4. HepG2 cells were transiently transfected with expression vectors for ERα-VP16 and the peptide-Gal4 fusion proteins. In addition, a luciferase reporter construct under the control of five copies of a Gal4 upstream enhancer element was also transfected along with a pCMV–β-galactosidase (β-Gal) vector to normalize for transfection efficiency. Transfection of the Gal4 DBD alone is included as control. Cells were then treated with various ligands (100 nM) as indicated and assayed for luciferase and β-Gal activity. Normalized response was obtained by dividing the luciferase activity by the β-Gal activity. Transfections were performed in triplicate, and error bars represent standard error of the mean (SEM). Triplicate transfections contained 1000 ng of ERα-VP16, 1000 ng of 5× Gal4-tata-Luc, 1000 ng of peptide-Gal4 fusion construct, and 100 ng of pCMV–β-Gal (*10*).

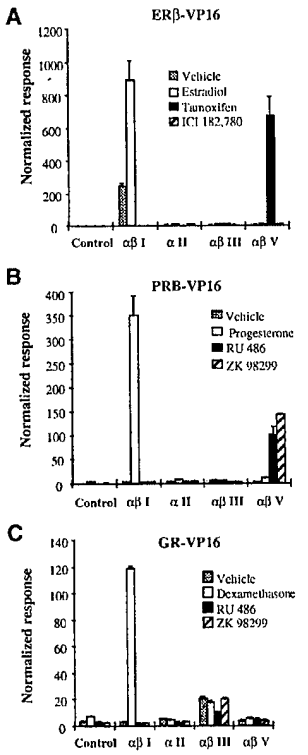

Fig. 3. Specificity of nuclear receptor–peptide interactions. Two-hybrid experiments were performed as in Fig. 2 between peptide-Gal4 fusion proteins and either (**A**) ERβ-VP16, (**B**) PRB-VP16, or (**C**) GR-VP16 (*15*). RU 486 and ZK 98299 are pan-antagonists of PRB and GR.

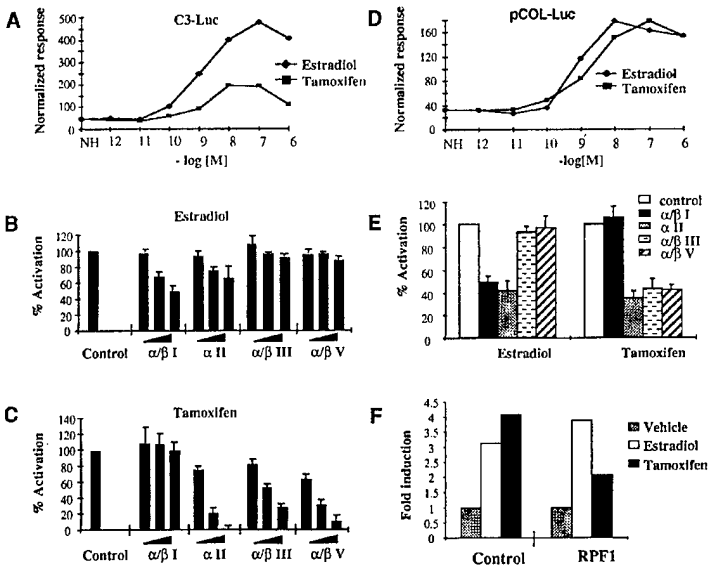

Fig. 4. Disruption of ERα-mediated transcriptional activity. **(A)** HepG2 cells were transfected with the estrogen-responsive C3-Luc reporter gene (*12*) along with expression vectors for ERα (*16*) and β-Gal and normalized as in Fig. 2. Cells were induced with either estradiol or tamoxifen as indicated and analyzed for luciferase and β-Gal activity. NH, no hormone. **(B)** HepG2 cells were transfected as in (A) except that expression vectors for peptide-Gal4 fusions were included as indicated. Control represents the transcriptional activity of estradiol (10 nM)–activated ERα in the presence of the Gal-4 DBD alone and is set at 100% activity. Increasing amounts of input plasmid for each Gal4-peptide fusion are also shown (▲) with the resulting transcriptional activity presented as percentage of activation of control. Data are averaged from three independent experiments (each performed in triplicate) with error bars representing SEM. Triplicate transfections contained 1000 ng of C3-Luc, 1000 ng of ERα expression vector, 100 ng of pCMV–β-Gal, and either 100, 500, or 1000 ng of peptide-Gal4 fusion construct. **(C)** Same as in (B) except that 4-OH tamoxifen (10 nM) was used to activate the receptor. **(D)** HepG2 cells were transfected with the AP-1–responsive collagenase reporter gene construct (pCOL-Luc) (*12*) and expression vectors for ERα and β-Gal. Cells were then induced with either estradiol or tamoxifen as indicated. **(E)** Same as (D), except that peptide-Gal4 fusion constructs were also transfected as indicated. Control represents the transcriptional activity of either estradiol- or tamoxifen (100 nM)–activated ER in the presence of the Gal4 DBD alone and is set at 100% activity. The transcriptional activity of estradiol and tamoxifen is shown in the presence of each Gal4-peptide fusion with the resulting transcriptional activity presented as percentage of activation of control. Triplicate transfections contained 1000 ng of pCOL-Luc, 1000 ng of ERα expression vector, 1000 ng of peptide-Gal4 fusion construct, and 100 ng of pCMV–β-Gal. Data are presented as in (B) and (C). **(F)** HeLa cells were transfected with the 1X-ERE-tata-Luc reporter gene along with expression vectors for ERα, β-Gal, and either RPF1 (pCDNA3-RPF1) or control vector [pcDNA3 (Invitrogen, Carlsbad, CA)]. Cells were induced with ligand (10 nM) as indicated. Data are presented as fold induction, which represents the ratio of ligand induced versus vehicle for each transfection.

demonstrate that, similar to α/β V, overexpression of RPF1 specifically represses tamoxifen-mediated partial agonist activity (Fig. 4F). However, the physiological importance of this activity remains to be determined. In summary, we have identified a series of peptide antagonists of ERα and hence validated additional target sites other than the ligand-binding pocket for drug discovery.

References and Notes

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6. Phage display was performed as described [L. A. Paige *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 3999 (1999)]. Immulon 4 96-well plates (Dynex Technologies, Chantilly, VA) were coated with streptavidin in $NaHCO_3$ buffer (pH 8.5) at 4°C for about 18 hours. Wells were blocked with bovine serum albumin (BSA) and then washed with TBST [10 mM tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20], and 2 pmol of biotinylated vitellogenin ERE was then added per well. Plates were washed with TBST, 3 pmol of baculovirus-purified ERα or ERβ (Pan Vera, Madison, WI) was then added, and plates were incubated at room temperature for 1 hour. Hormone was then added (1 μM) along with phage library (containing about 1.5×10^9 phage) in TBST and incubated at room temperature for 1 hour. Nonbinding phage were removed by washing with TBST. Bound phage were eluted in prewarmed (50°C) 50 mM glycine-HCL (pH 2.0). Eluent was neutralized by the addition of 200 mM Na_2HPO_4 (pH 8.5), and phage were amplified in *Escherichia coli* (DH5αF′). Affinity selection was repeated three times, and individual phage were isolated from either the second or third round of amplification. Peptide sequences were then deduced by DNA sequencing.
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10. HepG2 cells were maintained in modified Eagle's medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Life Technologies). Transfections were performed as described [J. D. Norris *et al.*, *J. Biol. Chem.* **270**, 22777 (1995)]. pCMV–β-Gal and 5× GAL4-tata-Luc were described previously [B. L. Wagner, J. D. Norris, T. A. Knotts, N. L. Weigel, D. P. McDonnell, *Mol. Cell. Biol.* **18**, 1369 (1998)]. Gal4 DBD-peptide fusions were created as follows: Peptide-coding sequences were excised from mBAX vector with Xho I–Xba I and subcloned into pM vector (Clontech, Palo Alto, CA) with a linker sequence to generate Sal I and Xba I sites for cloning. ERα-VP16 was generated by polymerase chain reaction (PCR) of human ERα-cDNA containing Eco RI sites flanking both 5′ and 3′ termini. The resultant PCR product was then subcloned into pVP16 (Clontech). All PCR products were sequenced to ensure the fidelity of the resultant construct. 17β-estradiol, 4-hydroxy-tamoxifen, and nafoxidine were purchased from Sigma.
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p300/CBP-mediated p53 acetylation is commonly induced by p53-activating agents and inhibited by MDM2

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The tumor suppressor p53 is activated in response to many types of cellular and environmental insults via mechanisms involving post-translational modification. Here we demonstrate that, unlike phosphorylation, p53 invariably undergoes acetylation in cells exposed to a variety of stress-inducing agents including hypoxia, anti-metabolites, nuclear export inhibitor and actinomycin D treatment. *In vivo*, p53 acetylation is mediated by the p300 and CBP acetyltransferases. Overexpression of either p300 or CBP, but not an acetyltransferase-deficient mutant, efficiently induces specific p53 acetylation. In contrast, MDM2, a negative regulator of p53, actively suppresses p300/CBP-mediated p53 acetylation *in vivo* and *in vitro*. This inhibitory activity of MDM2 on p53 acetylation is in turn abrogated by tumor suppressor p19^{ARF}, indicating that regulation of acetylation is a central target of the p53–MDM2–p19^{ARF} feedback loop. Functionally, inhibition of deacetylation promotes p53 stability, suggesting that acetylation plays a positive role in the accumulation of p53 protein in stress response. Our results provide evidence that p300/CBP-mediated acetylation may be a universal and critical modification for p53 function.

Keywords: acetylation/CBP/MDM2/p300/p53

Introduction

The tumor suppressor p53 plays a critical role in human cancer formation. In response to a variety of stress signals, often associated with the progression of neoplastic diseases, p53 becomes activated and induces cell cycle arrest and/or programmed cell death (apoptosis). By eliminating damaged and potentially dangerous cells that might otherwise become cancerous, p53 suppresses tumor formation. In unstressed cells, p53 is latent and is maintained at low levels by targeted degradation mediated by its negative regulator, MDM2 (reviewed in Freedman *et al.*, 1999). The critical role of MDM2 in regulating p53 is best illustrated by a study carried out in mice where inactivation of p53 was shown to completely rescue the embryonic lethality caused by the loss of MDM2 function (Montes de Oca Luna *et al.*, 1995). MDM2 counteracts p53 tumor suppressor activity by physically binding to p53 and suppressing its transcriptional activity. MDM2 also functions as the p53 ubiquitin ligase and triggers its degradation (reviewed in Freedman *et al.*, 1999). This latter activity requires the Ring finger domain located at the C-terminus of MDM2 (Fang *et al.*, 2000), and may also involve the acetyltransferase p300, which binds both MDM2 and p53 (Grossman *et al.*, 1998). Therefore, MDM2 negatively regulates p53 by at least two independent mechanisms.

The activation and stabilization of p53 are thought to be mediated by specific protein modifications, with phosphorylation being the major focus of earlier studies (reviewed in Giaccia and Kastan, 1998; Appella and Anderson, 2000). Although the exact functions of specific phosphorylation events remain controversial, evidence indicates that they probably contribute to both the stabilization and activation of p53. For example, DNA-damaging agents activate phosphorylation at serine (Ser) 15 and Ser37, likely by a family of protein kinases including ATM and ATR (Canman *et al.*, 1998; Tibbetts *et al.*, 1999), and Ser20 by the Chk2 kinase (Hirao *et al.*, 2000; Shieh *et al.*, 2000). These phosphorylation events are believed to contribute to p53 stabilization by preventing the binding of MDM2 and rendering p53 more resistant to MDM2 (Shieh *et al.*, 1997; Unger *et al.*, 1999).

In addition to potentially regulating MDM2 binding, phosphorylation was also shown to modulate the transcriptional activity of p53. For example, phosphorylation at Ser15 stimulates p53 interaction with its transcriptional co-activators p300 and CBP, and a mutation that eliminates this phosphorylation leads to p53 transcriptional defects (Lambert *et al.*, 1998; Dumaz and Meek, 1999). However, the requirement for the aforementioned phosphorylation is probably not universal for p53 stabilization or activation. For example, inhibition of RNA polymerase II by actinomycin D leads to p53 stabilization and activation without invoking either Ser15 or Ser20 phosphorylation (Ashcroft *et al.*, 2000). Similarly, viral oncoprotein E1A-induced p53 activation is not accompanied by Ser15 phosphorylation (de Stanchina *et al.*, 1998). These results suggest that alternative pathways and/or modifications exist and play important roles in modulating p53 activation. One such possible pathway involves the tumor suppressor p19^{ARF}. Inappropriate expression of E1A and other cellular oncogenes, such as c-*myc*, leads to p53 activation through a p19^{ARF}-dependent pathway (de Stanchina *et al.*, 1998; Zindy *et al.*, 1998). p19^{ARF} functions, at least in part, by binding to MDM2 and neutralizing its activity (Pomerantz *et al.*, 1998; Zhang *et al.*, 1998). p19^{ARF} inhibits the p53 ubiquitin ligase activity of MDM2 *in vitro* (Honda and Yasuda, 1999), and sequesters MDM2 into nucleoli,

thereby preventing its nuclear export *in vivo* (Weber *et al.*, 1999). Because the ubiquitin ligase activity and the nuclear export of MDM2 appear to be essential for the degradation of p53 (Tao and Levine, 1999a,b), it is possible that by directly binding and inactivating MDM2, $p19^{ARF}$ bypasses the need for phosphorylation in p53 activation.

Another potential mechanism that may play a critical role in p53 activation is acetylation. Multiple lysine (Lys) residues in p53 are reported to be acetylated. *In vitro*, Lys320 can be acetylated by P/CAF (p300/CBP associated factor) (Liu *et al.*, 1999) and CBP (A.Ito and T.P.Yao, unpublished result), while Lys373 and Lys382 are acetylated by p300 and CBP (Sakaguchi *et al.*, 1998; Liu *et al.*, 1999). At least two additional lysine residues (Lys370 and Lys381) are acetylated by CBP (A.Ito and T.P.Yao, unpublished result). *In vivo* studies show that some of these sites are acetylated in response to DNA-damaging agents, demonstrating that acetylation is a bona fide modification for p53 (Sakaguchi *et al.*, 1998; Liu *et al.*, 1999). However, despite the observation that acetylation can stimulate p53 DNA binding activity *in vitro* (Gu and Roeder, 1997; Liu *et al.*, 1999), the exact function of acetylation and the identities of the p53 acetylases that modify these sites *in vivo* remain to be established.

p300 and its family member CBP are the candidate *in vivo* p53 acetylases. p300 and CBP were originally discovered as transcriptional co-activators that play critical roles in integrating multiple signal-dependent transcription events (reviewed in Goodman and Smolik, 2000). *In vivo*, genetic experiments have clearly demonstrated essential roles for p300 and CBP in normal embryonic development (Tanaka *et al.*, 1997; Yao *et al.*, 1998; Kung *et al.*, 2000). More recent analyses have indicated that p300 and CBP may have specific roles in tumor suppression pathways. p300 mutations were recently found in many types of tumor (Gayther *et al.*, 2000) and mutation of human CBP causes Rubinstein–Taybi syndrome (RTS), which leads to an increased risk of cancers (reviewed in Giles *et al.*, 1998). The human genetic evidence was further substantiated by the analysis of CBP knockout mice, which also display a higher risk of tumors of hematopoietic origin (Gayther *et al.*, 2000; Kung *et al.*, 2000). Interestingly, many of the p300 mutations identified from tumors actually result in the loss of acetyltransferase activity (Gayther *et al.*, 2000), suggesting that the ability of p300 and CBP to acetylate one or more cellular proteins may be critical for their functions in growth control. The fact that p300 and CBP play important roles in p53 transcriptional activity (Gu *et al.*, 1997; Lill *et al.*, 1997) suggests that p53 might be a critical substrate of p300/CBP in mediating tumor suppression.

In this report, we present evidence that acetylation is a common modification associated with p53 activation in response to all p53-activating agents tested. We also establish that, *in vivo*, p300 and CBP can function as p53 acetylases and positively regulate p53 acetylation status, while MDM2 suppresses p53 acetylation. Consistent with p53 acetylation being a critical target of MDM2, we show that the tumor suppressor $p19^{ARF}$ can specifically inhibit the ability of MDM2 to negatively regulate p53 acetylation. Lastly, we provide evidence that inhibition of deacetylation increases the half-life of p53, suggesting that acetylation plays a role in p53 stability. Our results provide strong evidence that acetylation is a tightly regulated event and may be a universal and critical modification for p53 function.

Results

To initially address the potential importance of acetylation, we first determined whether p53 becomes acetylated in response to various environmental or cellular insults that are known to activate and stabilize p53. We used an antibody that specifically recognizes acetylated p53 at Lys382 (Sakaguchi *et al.*, 1998) or an antibody that recognizes a cluster of acetylated lysine residues (pan-acetylated p53, including lysines 370, 372, 373, 381 and 382) to confirm specific acetylation. Because in most cases both antibodies give very similar results in assessing p53 acetylation *in vivo* (for example, see Figure 2), the majority of results in this report are based on the analysis of Lys382 acetylation.

p53 acetylation is commonly induced by multiple p53-activating agents

Consistent with earlier reports, DNA damaging agents, such as UV irradiation (Figure 1A) and the DNA strand breakers camptothecin and *cis*-platinum (data not shown), all efficiently induce p53 acetylation. However, in the earlier reports the deacetylase inhibitor trichostatin A (TSA) was added during treatment to enhance the acetylation signal. This treatment prevents analysis of the kinetics of p53 acetylation (Sakaguchi *et al.*, 1998). To address this issue, we carried out the experiment in the absence of TSA. As shown in Figure 1A, p53 acetylation is a transient event and, after an initial increase, the abundance of acetylated p53 decreased due to the activity of a putative p53 deacetylase. Importantly, the kinetics of p53 acetylation paralleled that of its stabilization, suggesting that acetylation may play a role in p53 activation (Figure 1A).

To investigate further the involvement of acetylation in p53 activation, we examined whether p53-activating agents other than DNA damaging treatment can induce p53 acetylation. Many different types of cellular and environmental insult are capable of activating p53. Here we tested hypoxia, oxidative stress, blocking of nuclear export by leptomycin B (LMB) and depletion of ribonucleotides pools by *n*-phosphonacetyl-L-aspartate (PALA) (reviewed in Giaccia and Kastan, 1998; Freedman *et al.*, 1999). All of these treatments are capable of activating and stabilizing p53. As shown in Figure 1B–E, these agents stabilized p53 and, in every single case, p53 became acetylated. Importantly, treatment with the proteasome inhibitor LLnV, despite its ability to increase total p53 levels, did not result in increased acetylation, demonstrating that the acetylation signals detected were specific and not simply a consequence of higher protein levels (LL in Figure 1B and C). Inhibition of RNA polymerase II by actinomycin D is unique and different from DNA damaging or hypoxia treatment as it activates p53 without triggering phosphorylation of Ser15 or Ser20 (Ashcroft *et al.*, 2000, and data not shown). Figure 1F shows that actinomycin D still efficiently

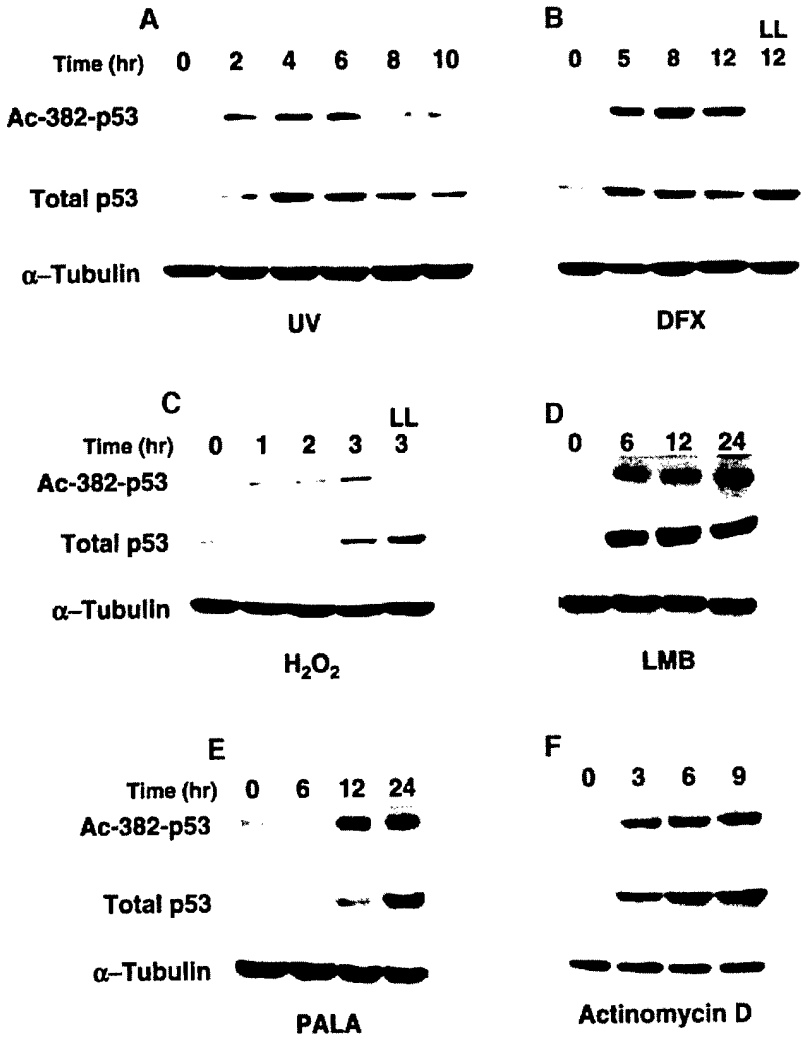

Fig. 1. p53 acetylation induced by multiple p53-activating agents. A549 cells were treated with (**A**) UV-B (100 J/m^2), (**C**) H_2O_2 (1 mM) or proteasome inhibitor LLnV (LL, 10 μM) for 3 h, (**D**) LMB (10 ng/ml) or (**F**) actinomycin D (5 nM). A549 and MCF7 cells (data not shown) were exposed to (**B**) deferoxamine mesylate (DFX) to mimic hypoxia (100 μM) or proteasome inhibitor LLnV (LL, 10 μM) for 12 h. WI-38 cells were exposed to (**E**) PALA (100 μM). (A–F) All cells were harvested at the times indicated. All cells contain wild-type p53. Total p53, acetylated p53 and the internal control α-tubulin levels were assessed by western blotting with α-p53 monoclonal antibody (middle panel), α-acetylated p53 (Lys382) (top panel) and α-tubulin monoclonal antibody (bottom panel), respectively. All treatments were carried out without the use of TSA, except for the DFX experiment where 5 μM of TSA was added to cells.

induced p53 acetylation, distinguishing acetylation from phosphorylation during p53 activation. Altogether, these results demonstrate that p53 becomes acetylated in response to all p53-activating agents tested in this study, and further indicate that acetylation is a common modification associated with p53 activation.

p300 and CBP function as p53 acetylases in vivo

Prime candidates for the p53 acetylases are p300 and its family member CBP. Both p300 and CBP can acetylate p53 *in vitro* (Gu and Roeder, 1997; Sakaguchi *et al.*, 1998; Liu *et al.*, 1999; Figure 4A). However, it is not known whether these acetyltransferases can function as p53 acetylases *in vivo*. To address this issue, we determined whether overexpression of p300 or CBP can induce the specific acetylation of endogenous p53. As shown in Figure 2A, overexpression of wild-type p300 in human 293T cells significantly induced p53 acetylation levels as illustrated by antibodies against acetylated Lys382 (top panel), pan-acetylated p53 (middle panel) or acetylated Lys373 (data not shown). The acetylation of p53 depends on the acetyltransferase activity of p300, as an acetylase-deficient point mutant (DY mutant) derived from a human tumor mutation (C.-H.Lai and T.-P.Yao, manuscript in preparation) failed to induce p53 acetylation. In contrast to p300 or CBP, the expression of P/CAF, which acetylated p53 at Lys320 *in vitro*, did not result in acetylation detectable by the antibodies used in this study (Figure 2A). In p53-null H1299 cells, co-expression of wild-type p53 and p300 also led to specific acetylation of the transfected

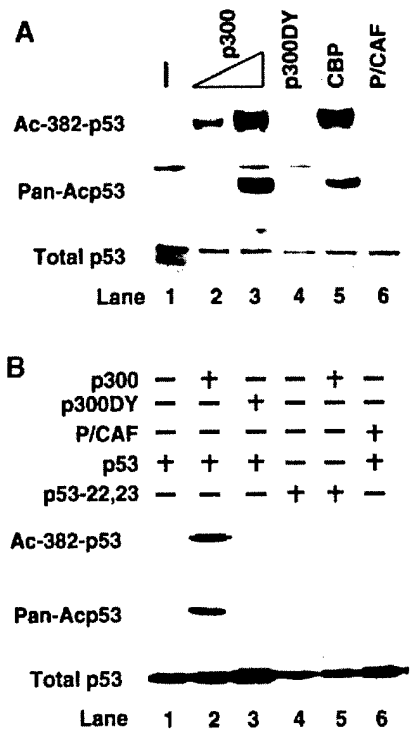

Fig. 2. Acetylation of p53 by p300 and CBP *in vivo*. (**A**) 293T cells were transfected with p300 (lanes 2 and 3), acetyltransferase-deficient p300 DY mutant (lane 4), CBP (lane 5) or PCAF (lane 6), and levels of endogenous acetylated p53 were assessed by either antibody specific for acetylated Lys382 (Ac-382-p53) or antibody against a cluster of acetylated lysines (Pan-Acp53; see text for details). The protein levels of p300, p300 DY, CBP and P/CAF were all comparable (data not shown). (**B**) H1299 cells ($p53^{-/-}$) were transfected with expression plasmid for wild-type p53 alone (lane 1), or co-transfected with either p300 (lane 2), p300 DY mutant (lane 3) or PCAF (lane 6). H1299 cells were also transfected with an expression plasmid for p53(22,23) p300 binding mutant alone (lane 4) or co-transfected with p300 (lane 5). For (A) and (B), cell extracts were prepared (36 h post-transfection) and the detection of acetylated p53 (Ac-382-p53 and Pan-Acp53) or total p53 was determined as described in the legend to Figure 1.

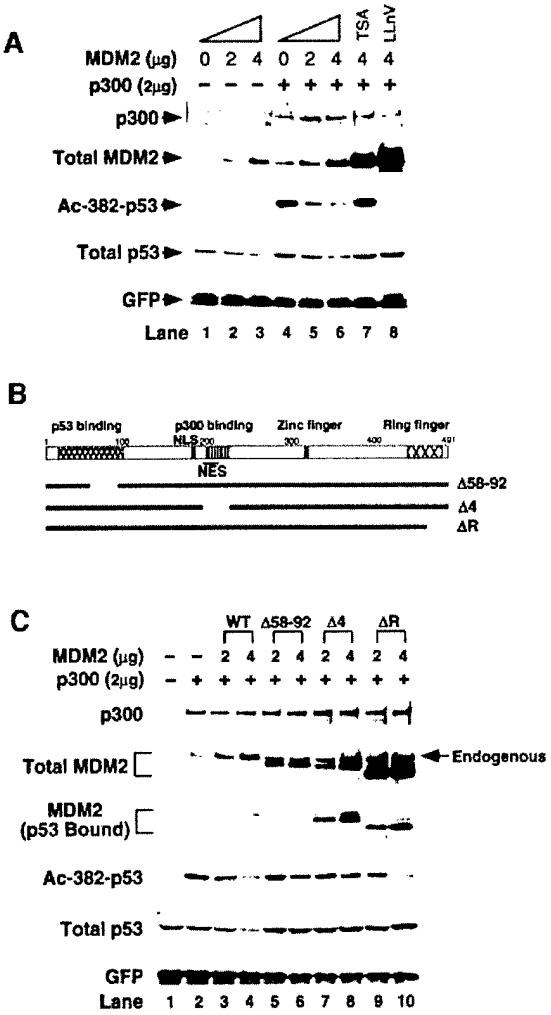

Fig. 3. Suppression of p300-dependent p53 acetylation by MDM2. (**A**) H1299 cells were transfected with expression plasmid for wild-type p53 and internal control GFP (lane 1), and co-transfected with either MDM2 (lanes 2 and 3), c-myc-tagged p300 (lane 4), or MDM2 and c-myc-tagged p300 (lanes 5–8). Cells were also treated 24 h post-transfection with either the deacetylase inhibitor TSA (5 μM) (lane 7) or the proteasome inhibitor LLnV (10 μM) (lane 8) for 12 h. Cell extracts were prepared (36 h post-transfection) and the level of acetylation (third panel) and total p53 protein (fourth panel) were determined by western blotting as described for Figure 1. (**B**) Schematic diagram of MDM2 deletion mutants used in (C). (**C**) H1299 cells were transfected with p53 wild-type and internal control GFP (lane 1), or co-transfected with c-myc-tagged p300 (lane 2), or c-myc-tagged p300 and the indicated amounts of MDM2 wild type (lanes 3 and 4), Δ58–92 mutant (lanes 5 and 6), Δ4 mutant (lanes 7 and 8) or ΔR mutant (lanes 9 and 10). p53 protein and acetylation levels were determined as described in (A). p300 levels were determined by either anti-myc (A14, Santa Cruz) (A) or by anti-p300 (RW128) (C). Both antibodies yielded similar results.

p53 species similar to that observed for the endogenous p53 (Figure 2B, lane 2). Again, the acetylation of the transfected p53 required wild-type p300 acetyltransferase activity (Figure 2B, lane 3). Importantly, a p300-binding-deficient p53 mutant could not be acetylated when co-expressed with p300 (lanes 4–5). This result indicates that direct binding between p53 and p300 is necessary for efficient acetylation and provides further evidence that p53 acetylation is mediated directly by p300 *in vivo*. Identical results were observed when CBP was evaluated for its role in p53 acetylation (Figure 2A, lane 5, and data not shown).

MDM2 suppresses p300/CBP-dependent p53 acetylation in vivo

The results presented thus far provide strong evidence that p53 is acetylated by its positive regulators p300 and CBP in response to a variety of signals. If acetylation plays a critical role in p53 function, it is likely that factors that negatively regulate p53 activity might interfere with this process. MDM2 is the most important p53 negative regulator and it also interacts with p300 (Grossman *et al.*, 1998). These observations prompted us to ask whether MDM2 has the capacity to regulate the acetylation status of p53. As shown in Figure 3A, overexpression of MDM2 effectively reduced p300-dependent p53 acetylation in a

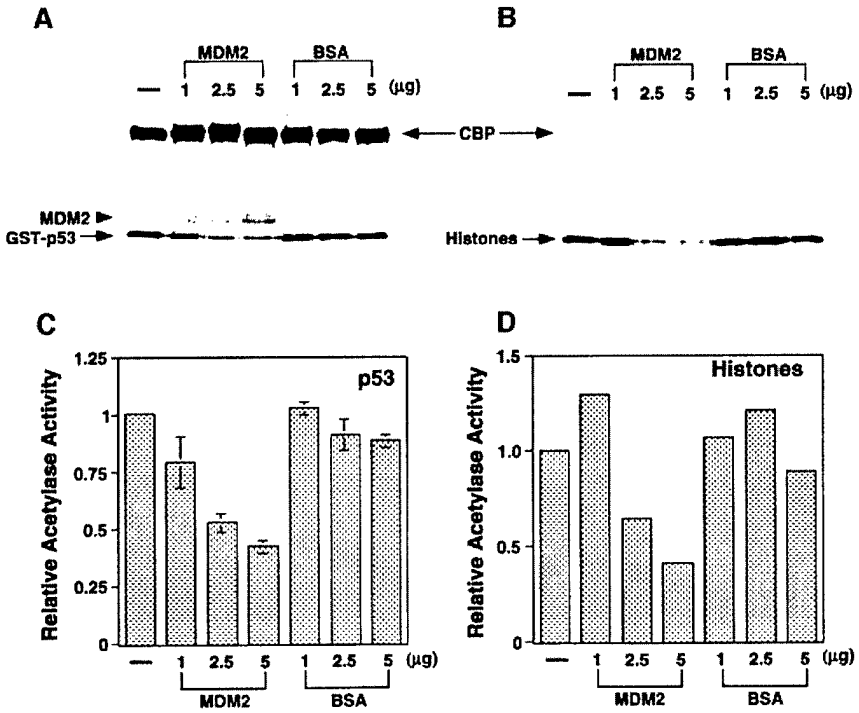

Fig. 4. Suppression of CBP acetyltransferase activity by MDM2 *in vitro*. (A and B) GST–p53 (**A**) or core histones (**B**) were acetylated by recombinant CBP in the presence of the indicated amounts of MDM2 or BSA, and analyzed by SDS–PAGE followed by autoradiography. Film was exposed (A) overnight and (B) for 3 h. Acetylated p53 and histone are indicated with arrows. Note that the level of acetylated p53 and acetylated histone decreases in the presence of MDM2. Acetylated MDM2 is marked with an arrowhead (A). (C and D) The intensity of the acetylated GST–p53 (**C**) or histones (**D**) was quantified by phosphoimager analysis and plotted. The intensity of acetylated GST–p53 or histones in the absence of MDM2 or BSA was set as 1. (C) reflects the average of three experiments, while (D) reflects the average of two experiments.

dose-dependent manner (lanes 4–6). Of note, MDM2 overexpression does not affect the protein levels of transfected p300 (Figure 3A, top panel), supporting a direct effect of MDM2 on p53 acetylation. To rule out the possibility that the decrease in acetylation was caused by a corresponding decrease in p53 protein levels triggered by MDM2, the proteasome inhibitor LLnV was added to the culture to block p53 degradation. This treatment led to the stabilization of p53. Despite high protein levels, p53 remained non-acetylated in the presence of MDM2 (Figure 3A, lane 8). This result demonstrates that MDM2 can reverse the p53 acetylation induced by p300. In contrast to LLnV treatment, the deacetylase inhibitor TSA effectively abrogated the effect of MDM2 and restored p53 acetylation (Figure 3A, compare lanes 6 and 7), providing further evidence that MDM2 specifically modulated p53 acetylation. Interestingly, TSA treatment also increased p53 protein levels, suggesting the possibility that inhibition of p53 deacetylation promoted p53 stability (see below).

To study further how MDM2 suppresses p53 acetylation, we analyzed a series of MDM2 mutants with specific functional domains deleted (Figure 3B). Specifically, we tested MDM2 mutants that are deficient in p53 binding (Δ58–92) (Chen *et al.*, 1993), p300 binding (Δ4, amino acids 192–222) (Grossman *et al.*, 1998), or defective in ubiquitin ligase activity (ΔR, deletion of the Ring domain). As shown in Figure 3C, after transfection into H1299 cells, all these MDM2 variants were expressed (second panel). However, when compared with wild-type MDM2 (lanes 3–4), both the p53 binding mutant (Δ58–92, lanes 5–6) and the p300 binding mutant (Δ4, lanes 7–8) were defective as they only weakly suppressed p53 acetylation even when expressed at higher levels (Figure 3C, Ac-382). Importantly, both mutants are also deficient in degrading p53, further suggesting a functional link between p53 acetylation and stability. In contrast, the Ring domain mutant inhibited p53 acetylation to a level similar to that of wild-type MDM2 (ΔR, lanes 9–10). These results indicate that physical binding to both p53 and p300 is required for full activity of MDM2 to repress p53 acetylation. The Ring domain, which is essential for degrading p53 (Fang *et al.*, 2000), is dispensable for this function. From this set of experiments, we conclude that MDM2 can actively repress p300-mediated p53 acetylation *in vivo* and that this activity requires physical binding to both p53 and p300.

MDM2 suppresses CBP acetyltransferase activity in vitro

In principle, MDM2 could repress p53 acetylation either by directly suppressing p53 acetylation or by promoting p53 deacetylation. To address these possibilities, we first determined whether MDM2 could directly inhibit

p300/CBP-mediated p53 acetylation *in vitro*. As shown in Figure 4A, although recombinant MDM2 had no effect on CBP auto-acetylation, it efficiently inhibited p53 acetylation in a dose-dependent manner (Figure 4A and C). As this inhibition was not sensitive to the deacetylase inhibitor TSA (data not shown), MDM2 likely interfered with the acetyltransferase activity of CBP rather than functioning as a p53 deacetylase. Interestingly, while suppressing p53 acetylation, MDM2 itself became acetylated by CBP (Figure 4A, arrowhead). The functional importance of this acetylation is not yet clear. To determine whether the effect of MDM2 on CBP was specific to p53 acetylation, we tested whether MDM2 could suppress CBP-mediated histone acetylation. As shown in Figure 4B and D, MDM2 was able to suppress the acetylase activity of CBP towards core histones as well. In contrast, under the same experimental conditions, MDM2 had no apparent suppressive effect on another acetyltransferase, P/CAF (data not shown). Thus, MDM2 can specifically suppress CBP-mediated p53 and core histone acetylation *in vitro*. These observations suggest that the ability of MDM2 to repress p53 acetylation *in vivo* works, at least in part, by suppressing the acetyltransferase activity of p300 and CBP.

Tumor suppressor p19^{ARF} reverses the inhibition of p53 acetylation by MDM2

p19^{ARF} induces p53 activation by negatively regulating MDM2. This activity is proposed to be mediated by inactivating p53 E3 ligase activity of MDM2. Analysis of the role of MDM2 in p53 acetylation suggests an alternative possibility that p19^{ARF} might function by antagonizing the activity of MDM2 toward p53 acetylation. To examine this possibility, we determined whether co-expression of p19^{ARF} and MDM2 could neutralize the latter's ability to repress p300-dependent p53 acetylation. As shown in Figure 5, in the absence of p19^{ARF}, co-transfection of MDM2 efficiently repressed p53 acetylation and induced its degradation in H1299 cells (compare lanes 2 and 3). However, upon co-expression of p19^{ARF}, both p53 acetylation and protein levels were restored (lanes 4–5). Importantly, a p19^{ARF} mutant, which does not bind MDM2 (C65, Zhang and Xiong, 1999) failed to suppress MDM2 in this assay (lane 6). Altogether, these results demonstrate that p19^{ARF} can abrogate the ability of MDM2 to suppress p53 acetylation. The correlation between p53 protein level and acetylation level in response to MDM2 and p19^{ARF}, however, does suggest that acetylation might influence p53 stability.

An increased level of total and p53-bound MDM2 was also observed when p19^{ARF} was co-expressed (Figure 5, MDM2 panels, lanes 4–5). This might be due to the inhibition of MDM2 auto-ubiquitylation (Fang *et al.*, 2000) and, consequently, the stabilization of MDM2. Importantly, despite the high levels of MDM2 associated with p53, MDM2 in this complex did not show appreciable repression toward p53 acetylation, supporting the idea that p19^{ARF} dominantly inhibits the activity of MDM2 in this assay. This observation also suggests that p19^{ARF} restores p53 acetylation and protein levels without dissociating MDM2 from p53. This set of results demonstrates that, in addition to inhibiting MDM2 as a p53 ubiquitin ligase, p19^{ARF} is also capable of inactivating MDM2 in suppressing p53 acetylation, providing further evidence that acetylation is a modification regulated by a p300/CBP–MDM2–p19^{ARF} feedback loop in the p53 network.

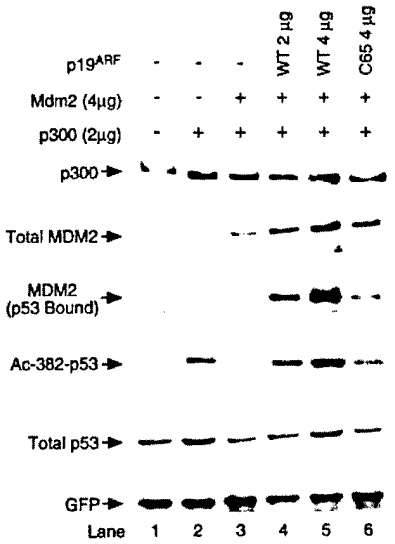

Fig. 5. p19^{ARF} reverses the inhibition of p53 acetylation by MDM2. H1229 cells were transfected with either expression plasmid for p53 wild-type and internal control GFP (lane 1) or co-transfected with c-myc-tagged p300 (lanes 2–6), in combination with MDM2 and p19 expression plasmid as indicated. Analysis of p53 protein and acetylation levels was carried out as described for Figures 1 and 2. Note that expression of p19 effectively neutralized the effects of MDM2 on p53 acetylation (lanes 4 and 5). p300 levels were determined by RW128.

Inhibition of p53 deacetylation promotes p53 stability

The results presented so far support the idea that acetylation is a common modification regulated by a network of critical regulators of p53 function. In principle, acetylation could contribute to p53 stabilization and/or p53 activity. Several observations from our study suggest the possibility that acetylation may regulate p53 stability. First, there was a positive correlation between the kinetics of p53 protein levels and its acetylation levels in response to DNA damage (Figure 1A). Secondly, p19^{ARF} concomitantly restored p53 protein and acetylation levels, which were negatively regulated by MDM2 (Figures 3 and 5). Lastly, treatment with the deacetylase inhibitor TSA seemed to result in higher p53 protein levels (Figure 3A). If acetylation were important for p53 stabilization, one would predict that TSA treatment should delay the normal rate of degradation by preventing p53 deacetylation. To test this hypothesis, p53 stability was determined following its activation by UV irradiation. As shown in Figure 6A (top panel), TSA treatment effectively inhibited the p53 deacetylase and increased the levels of acetylated p53 in A549 cells. Importantly, the apparent half-life of p53 was dramatically increased in the presence of TSA, suggesting that acetylated p53 is more stable (Figure 6A, middle panel, and B). In contrast, the same treatment did not affect the half-life of actin (Figure 6A, bottom panel), indicating that TSA did not have a general positive effect on protein

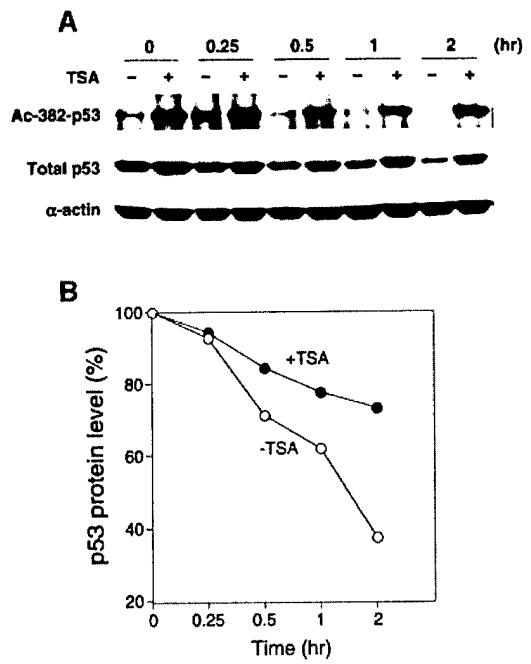

Fig. 6. Inhibition of deacetylase promotes p53 stabilization. (**A**) A549 cells were exposed to UV-B (50 J/m^2) in the presence (+) or absence (–) of TSA (5 μM). Four hours post-irradiation, cyclohexamide (10 μg/ml) was added to inhibit new p53 protein synthesis (designated 0 h). Cells were harvested at the time-points indicated after cyclohexamide treatment. Acetylated p53 (top panel) and total p53 (middle panel) were determined. Note that total p53 level and acetylation levels are significantly higher in the presence of TSA. As a control, direct western blotting with an α-actin polyclonal antibody also assessed actin levels (lower panel). (**B**) The band intensity of p53 protein levels was measured by NIH imaging software and calculated against the amount of p53 present at time point 0, which was set at 100%. Results are given in the presence (filled circles) or absence (empty circles) of TSA treatment.

stability. This result indicates that one function of specific p53 acetylation is to increase its stability.

Discussion

In this report, we show that p53 becomes acetylated in response to all p53-activating agents tested (Figure 1). Together with the recent report that p53 acetylation increases as fibroblasts senesce (Pearson *et al.*, 2000), these results clearly establish acetylation as a common modification that invariably accompanies p53 activation. This is in contrast to the two well studied phosphorylation events on Ser15 and Ser20, which are activated only by a select few agents (Ashcroft *et al.*, 2000), and further illustrates a unique requirement for acetylation in p53 activation. Although the complete function of p53 acetylation remains to be firmly established, we provide evidence that acetylation may at least contribute to p53 stability. Two recent reports have suggested that acetylation is important for p53 to suppress oncogenic ras-induced transformation (Pearson *et al.*, 2000) and to induce metaphase chromosome fragility (Yu *et al.*, 2000), adding more evidence for the functional significance of p53 acetylation. The findings that p300/CBP acetyltransferases and p19^{ARF} promote p53 acetylation *in vivo*, while MDM2 inhibits acetylation, lend support to the idea that acetylation is an important modification targeted by both positive and negative regulators critical to p53 tumor suppressor activity.

Reversible acetylation was originally identified in histones and was thought to be important for transcriptional activity (Wade *et al.*, 1997). However, a growing number of non-histone proteins are now being reported as targets of acetylation (reviewed in Kouzarides, 2000). Although in most cases the function of acetylation remains to be firmly established, analysis of E2F1 and myoD indicates that P/CAF-mediated acetylation appears to increase E2F1 stability (Martinez-Balbas *et al.*, 2000) and contribute to myoD activity (Sartorelli *et al.*, 1999). Similarly, p300/CBP-dependent GATA-1 acetylation has been shown to be critical for GATA-1 function (Boyes *et al.*, 1998). In this report, we further show that MDM2 may be an acetylated protein as well (Figure 4A). Together with the demonstration that acetylation of p53 is tightly regulated and is important for p53 stability, these various lines of evidence support the hypothesis that acetylation is a prominent and likely general regulatory modification used to modulate protein function.

We have presented evidence that p300 and CBP are able to acetylate p53 and are likely to be the key p53 acetylases *in vivo*. Biochemical and genetic experiments indicate that p300 and CBP levels are limited in cells (reviewed in Goodman and Smolik, 2000), and apparently, they cannot support endogenous p53 acetylation under normal conditions. In theory, high levels of p300/CBP in the transfection setting should increase the probability of complex formation with p53. Moreover, transfection itself probably triggers some DNA damage response. These two factors together may contribute to p53 acetylation upon p300/CBP overexpression (Figure 2). Consistent with this idea, we have shown that a direct interaction between p300/CBP and p53 is necessary for efficient p53 acetylation (Figure 2B). Under normal physiological settings, it is likely that p53 and p300/CBP complexes are induced in response to activating signals. Consistent with this idea, we have found that mutations that eliminate phosphorylation at Ser15 but not Ser20 significantly reduced p53 acetylation *in vivo* (our unpublished observation). As Ser15 phosphorylation stimulates p53 binding to p300/CBP (Lambert *et al.*, 1998), this result provides evidence that specific phosphorylation on Ser15 could be one activation step leading to p53–p300/CBP complex formation and subsequent p53 acetylation by p300/CBP. Ser15 phosphorylation, however, is not the only mechanism that can lead to p53 acetylation. Actinomycin D does not induce Ser15 phosphorylation (Ashcroft *et al.*, 2000), yet it is a powerful agent in triggering p53 acetylation (Figure 1F). This result suggests a more general and unique requirement for acetylation than some specific phosphorylation events during p53 activation. The mechanism by which actinomycin D induces p53 acetylation without Ser15 phosphorylation, however, remains unknown.

Our results show clearly that MDM2 can suppress p300/CBP-mediated p53 acetylation *in vitro* and *in vivo*. There are at least four possible mechanisms that may explain this

observation. First, MDM2 binds and inactivates p300/CBP acetyltransferase activity. This possibility is supported by our result that a p300-binding-deficient MDM2 mutant is defective in this activity. Secondly, p300/CBP and MDM2 bind to non-identical but overlapping regions at the N-terminus of p53. It is possible that high levels of MDM2 bind p53 and displace p300/CBP, thereby inhibiting p53 acetylation. This mechanism, however, may not explain how MDM2 suppresses histone acetylation, as there is no evidence that MDM2 binds histones. The observation that $p19^{ARF}$ restores p53 acetylation without dissociating MDM2 from p53 is also inconsistent with this model (Figure 5). Thirdly, MDM2 can interact directly with p300/CBP and itself becomes acetylated (Figure 4A). It is possible that MDM2 serves as a substrate competitor and thereby suppresses p53 acetylation. Further studies will be needed to verify the acetylation of MDM2 *in vivo* and the importance of this acetylation. Lastly, although MDM2 inhibits p53 acetylation by CBP directly *in vitro*, we could not eliminate the possibility that other mechanisms may also contribute to the suppression of p53 acetylation *in vivo*. For instance, MDM2 could stimulate deacetylation by recruiting a p53 deacetylase. The observation that TSA can completely abrogate the inhibitory effect of MDM2 on p53 acetylation (Figure 3A) and that MDM2 interacts with a specific deacetylase (A.Ito and T.P.Yao, unpublished result) is consistent with this possibility. Regardless of which mechanism is correct, our results clearly demonstrate that MDM2 is able to suppress p53 acetylation *in vivo* and *in vitro*.

Our analysis of MDM2 also reveals that MDM2 suppresses the core histone acetylation induced by p300/CBP. It has been hypothesized that p300 and CBP activate transcription by acetylating histones. The inhibitory activity of MDM2 on histone acetylation provides a biochemical mechanism to explain how MDM2 can inhibit p53 transactivation potency. In this scenario, the recruitment of MDM2 to the p53–p300 or p53–CBP complexes on target chromatin inhibits histone acetylation and thereby represses p53-dependent transcription.

By binding to MDM2, $p19^{ARF}$ plays a critical role in p53 activation. This activity of $p19^{ARF}$ was attributed, at least in part, to its ability to suppress the MDM2 E3 ligase activity toward p53 ubiquitylation (Honda and Yasuda, 1999). Our study now shows that $p19^{ARF}$ can also abrogate the inhibitory effect of MDM2 toward p53 acetylation *in vivo* (Figure 5). In fact, overexpression of $p19^{ARF}$ alone is sufficient to induce p53 acetylation (A.Ito, unpublished result). This observation adds a novel mechanism through which $p19^{ARF}$ regulates MDM2 activity and participates in tumor suppression. Two alternative hypotheses have been put forward to explain how $p19^{ARF}$ inhibits MDM2 activity. One proposes that $p19^{ARF}$ sequesters MDM2 in nucleoli and dissociates MDM2 from p53 (Weber *et al.*, 1999), while the other shows that p53–MDM2–$p19^{ARF}$ forms a tripartite complex in the nucleoplasm, where MDM2 is not active (Zhang and Xiong, 1999). Unexpectedly, we found that upon $p19^{ARF}$ expression, a dramatic increase in MDM2 was found to complex with p53. However, the MDM2 in this complex is not active in suppressing p53 acetylation (Figure 5, lanes 4 and 5). These observations are more consistent with the possibility of a tripartite complex formation wherein $p19^{ARF}$ dominantly inhibits the activity of MDM2 toward p53 acetylation. However, we have found that recombinant $p19^{ARF}$ does not interfere with the ability of MDM2 to suppress CBP-mediated p53 acetylation *in vitro* (A.Ito and T.P.Yao, unpublished observation). Further studies will be required to determine how $p19^{ARF}$ suppresses MDM2 activity in this ternary complex. Regardless, our study demonstrates that $p19^{ARF}$, in addition to regulating MDM2 ubiquitin ligase activity, can also suppress the activity of MDM2 towards p53 acetylation. These results suggest that all major regulators of p53 activity, including p300/CBP, MDM2 and $p19^{ARF}$, integrate different extracellular and intracellular signals to modulate p53 acetylation level and thereby its stability and activity.

What is the importance of p53 acetylation in relation to p53 function? It was first reported that acetylation increases p53 DNA binding activity *in vitro* (Gu and Roeder, 1997). However, analyses of p53 mutants that can not be acetylated do not reveal obvious defects in DNA binding *in vivo* (data not shown), suggesting that acetylation might have other functions. Three lines of evidence derived from this study suggest that acetylation functions, at least in part, by modulating p53 stability. First, there is positive correlation between endogenous p53 protein and acetylation levels upon normal p53 activation (Figure 1). Secondly, in analyzing the ability of various MDM2 mutants and $p19^{ARF}$ to regulate p53 acetylation (Figures 3 and 5), we found a similar correlation between p53 protein and acetylation levels. This conclusion is further supported by the observation that TSA can efficiently reverse the degradation of p53 induced by MDM2 (Figure 3A and data not shown). Thirdly, prevention of p53 deacetylation leads to a more stable p53 species (Figure 6). Given that acetylation is always accompanied by p53 stabilization (Figure 1), this correlative evidence strongly suggests that acetylation may be a modification that contributes to p53 stabilization. How does acetylation stabilize p53? Since both the acetyltransferase and ubiquitin-conjugating system through which p53 is targeted for degradation modify lysine, it is possible that acetylation protects lysine residues from being ubiquitylated. It was reported recently that several lysine residues located at the C-terminus target p53 for ubiquitylation and degradation (Rodriguez *et al.*, 2000). Importantly, these are the same lysine residues that can be acetylated by p300/CBP. These observations suggest the possibility that acetylation renders lysines unavailable for the ubiquitin-conjugating machinery, and thereby promotes p53 stability. If acetylation functions, at least in part, to inhibit ubiquitylation, reversible acetylation might have a more general role in regulating protein stability.

In conclusion, we propose that in response to cellular stresses, p53 becomes acetylated by the p300/CBP acetyltransferases. This modification requires either specific phosphorylation, such as at Ser15, or the activation of tumor suppressor $p19^{ARF}$. Acetylation leads to p53 stabilization and the subsequent induction of MDM2. MDM2 then in turn triggers p53 deacetylation followed by p53 inactivation and destruction. The modulation of p53 acetylation by CBP/p300, MDM2 and $p19^{ARF}$ suggests the existence of an intricate pathway regulating the acetylation equilibrium that is crucial to the tumor suppressor activity

of p53. Further characterization of the function of p53 acetylation will be critical for understanding the regulation of p53 tumor suppressor activity.

Materials and methods

Cell lines and transfection

A549, WI38, 293T and H1299 human cells were maintained in Dulbecco's modified Eagle's medium (DMEM). All cells were grown at 37°C in the presence of 10% fetal bovine serum and penicillin/streptomycin in a humidified atmosphere of 5% CO_2. A549 and WI38 cells have wild-type p53, while H1299 cells are devoid of any p53 expression. All transfections were performed by the calcium phosphate method as described previously (Yao *et al.*, 1992).

Plasmids

Wild-type human p53 cDNA was cloned into the *Bam*HI–*Xho*I site of pCDNA3. The mutant p53(22,33), which can not bind to p300 has been described previously (Gu *et al.*, 1997). The human MDM2 wild-type cDNA was cloned into the *Bam*HI–*Eco*RI site of pCDNA3. The MDM2 ΔR mutant cDNA was made by digesting wild-type pCDNA3-MDM2 with *Sal*I to delete the Ring domain (amino acids 442–491). The MDM2 Δ4 and Δ58–92 mutants were described previously (Chen *et al.*, 1993; Grossman *et al.*, 1998). The human p300-DY (Lys1399 converted to tyrosine) mutant was generated by site-direct mutagenesis and cloned into the pCMV vector. The full length mouse $p19^{ARF}$ and $p19^{ARF}$ N-terminal fragment (C65) (MDM2-binding-deficient mutant) were described previously (Zhang and Xiong, 1999).

Pulse–chase

A549 cells at 80–90% confluence were exposed to a 310 nm wavelength UV source. The deacetylase inhibitor TSA (Sigma) was added at a final concentration of 5 μM immediately after UV irradiation. Four hours after irradiation, cells were treated with 10 μg/ml of cyclohexamide to stop new p53 protein synthesis, and cells were then harvested at the indicated time points as described in Figure 6.

Immunoprecipitation and immunoblotting

Cells were lysed in buffer [20 mM Tris–HCl pH 7.6, 170 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM dithiothreitol (DTT)] supplemented with 5 μM TSA and protease inhibitors. For immunoprecipitation with anti-p53 antibody, equal amounts of lysate (containing 200–300 μg of total cellular protein) were incubated with 1 μg of goat anti-p53 antibody (Santa Cruz) and protein G–Sepharose (Pharmacia) for 3 h at 4°C. The use of goat antibody eliminates the heavy chain signal that co-migrates with p53 in subsequent immunoblotting. For immunoprecipitation with anti-p300 antibody, equal amounts of lysate (containing 100–150 μg of total cellular protein) were incubated with anti-p300 antibody (RW128) and protein G–Sepharose (Pharmacia) for 3 h at 4°C. When immunoprecipitation was not performed, 20–30 μg of total extracts were analyzed. Proteins were detected by chemiluminescent ECL kit (Amersham) with one of the following antibodies: anti-human p53 antibody (Ab-6, Calbiochem), anti-human acetylated (Lys382) p53 antibody (Calbiochem), anti-human MDM2 antibody (SMP14, Santa Cruz), anti-α-tubulin antibody (DM1A, Sigma), anti-c-myc antibody (A14, Santa Cruz), anti-p300 antibody (RW128, Eckner *et al.*, 1994), anti-green fluorescent protein (GFP) antibody (Boehringer Mannheim) or anti-actin antibody (C-11, Santa Cruz).

In vitro acetyltransferase assay

Recombinant CBP protein (1 μg) purified from baculovirus was pre-incubated with the indicated amounts of purified bacterially expressed MDM2 protein or bovine serum albumin (BSA) for 10 min at room temperature. After pre-incubation, substrates [1 μg of glutathione-*S*-transferase (GST)–p53 or histone] were added and incubated with 50 nCi [^{14}C]acetyl-coenzyme A in 30 μl of reaction buffer (50 mM Tris–HCl pH 8.0, 10% glycerol, 1 mM DTT, 100 μM EDTA, 1 mM phenylmethylsulfonyl fluoride) for another 45 min at 37°C. Acetylation was analyzed by SDS–PAGE followed by autoradiography, or by a phosphoimager.

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